Pathobiological aspects of duodenal adenomatosis in familial adenomatous polyposis

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Pathobiological aspects of duodenal adenomatosis in familial adenomatous polyposis

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Cover: WST-1 assay, Comet assay, CGH, normal duodenum, duodenal adenoma and carcinoma, E-cadherin staining and UGT polymorphisms
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It's the neoplastic lesions
which are called the adenomas
They're the ones that have the risk
of going on to carcinoma.

Now these chances are related
to their number, size and site
Their degree of cell dysplasia
and their histologic type.

In the stomach in the colon
duodenum and the rectum
They are tubular or villous
or a mixture of this spectrum.

Adenomas by the hundred
mean familial polyposis
A colectomy will help prevent
a carcinomatosis.

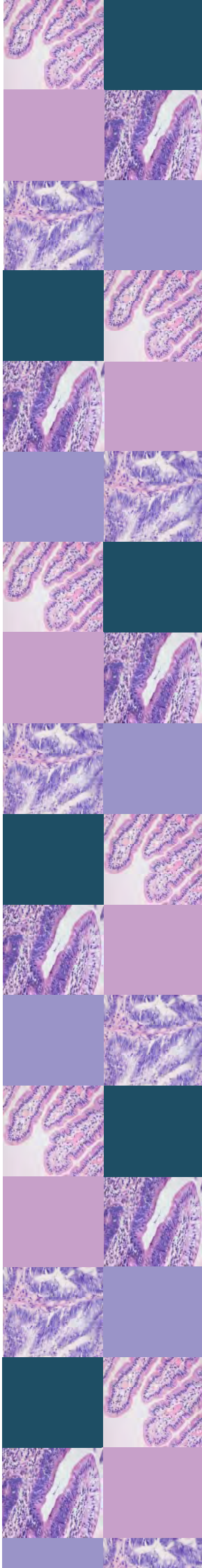
Tales of the Ampulla of Vater,

Leslie H. Sobin, M.D. 1994

Contents

Chapter 1

Introduction



Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder caused by germline mutations in the tumour suppressor gene a*denomatous polyposis coli* (*APC*) [1]. Patients develop hundreds to thousands of colorectal adenomas during the second and third decade of life. The risk of colorectal cancer at the age of 40-50 years is almost 100 per cent unless prophylactic colectomy is performed. The incidence of FAP is estimated at birth to be approximately one in 10,000 [2]. In about 10% of the patients, the disease is less severe with < 100 colorectal adenomas and a later onset of the disease. This variant is called attenuated FAP (AFAP) [3]. In addition to colorectal adenomas, several extra-colonic manifestations are observed, including desmoid tumours, osteomas, dental abnormalities, congenital hypertrophy of the retinal epithelium, lipomas, epidermoid cysts and upper gastrointestinal adenomas [4].

Adenomatous polyposis coli gene

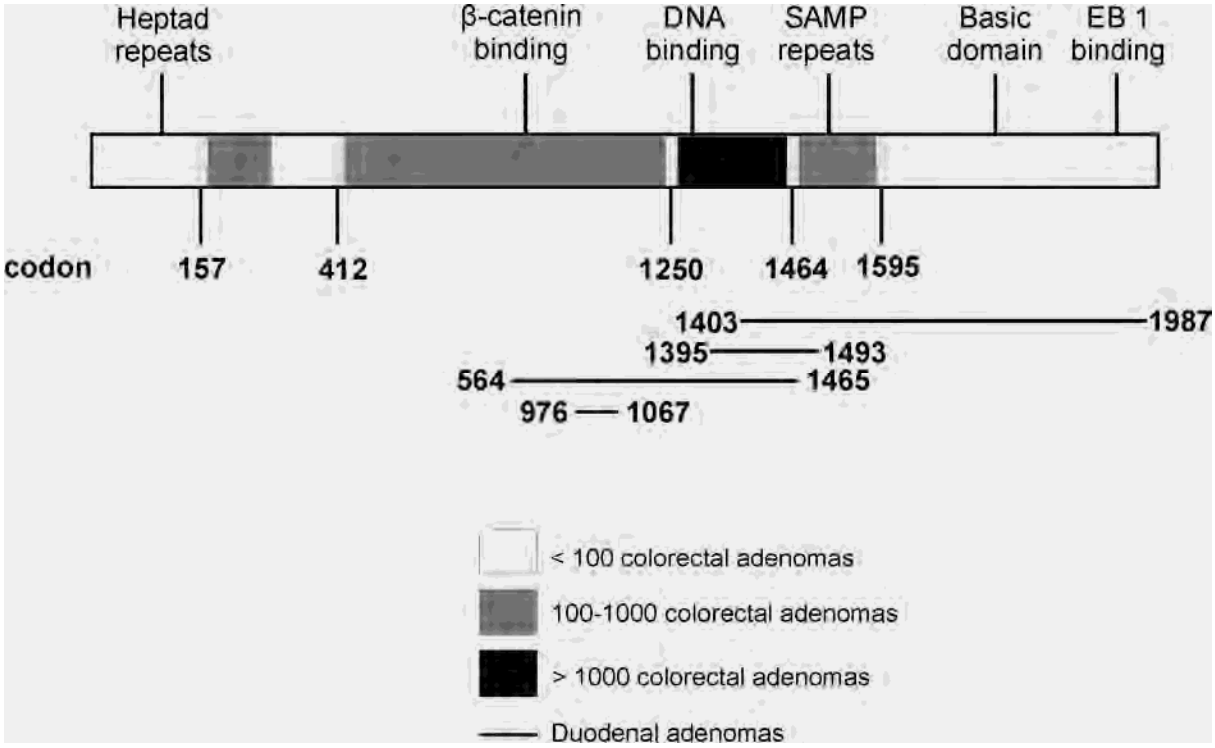
In 1987, the *adenomatous polyposis coli* (*APC*) gene was identified and found to have mutated in patients with FAP [1]. This gene is located on chromosome 5q21-22 and the coding region of this gene consists of 15 exons, encoding a protein of 2,842 amino acids. This large gene has a tumour suppressor function. The germline mutations found in patients with FAP often lead to a truncated non-functional protein [5]. The APC protein has a broad spectrum of functions, ranging from affecting cell adhesion and migration, to apoptosis, chromosomal segregation at mitosis and control of the Wnt signal transduction pathway. The Wnt signalling pathway regulates the phosphorylation and degradation of β-catenin [6]. β-catenin is a bi-functional protein which has a crucial role in cell-cell adhesion [7] and in the Wnt signalling pathway [8]. Loss of functional APC in tumourigenesis deregulates the signalling properties of β-catenin leading to cytosolic accumulation and nuclear translocation of the protein. In the nucleus, it activates transcriptional targets such as c-MYC [9] and cyclin D1 [10]. In cell adhesion, β-catenin is crucial to the normal functioning of E-cadherin [11]. Inactivation of APC function seems to underlie both tumour initiation and promotion [12]. In 30-50% of patients with FAP or AFAP, no

germline mutation is detected [13, 14]. However, 10-30% of these patients have a mutation in the *MYH* gene, another recently identified polyposis gene. Mutations in this gene cause a recessive form of colonic polyposis [15].

Genotype-phenotype correlation

The site of the *APC* mutations can be associated with the severity of the colonic phenotype. In the attenuated phenotype (< 100 colorectal adenomas), the mutations are found before codon 157, after codon 1595 and in the alternative spliced region of exon 9. The phenotypes with severe polyposis (> 1000 colorectal adenomas) are associated with mutations between the codons 1250 to 1464. An intermediate phenotype (100 – 1000 adenomas) is linked to the rest of the gene. Upper gastrointestinal tumours are seen both in patients with FAP as well as those with AFAP and several locations at the *APC* gene have been related to the development of duodenal adenomas. However, no large study has been performed to establish an association between germline *APC* genotype and the severity of duodenal polyposis [16].

***Figure 1**: The localisation of mutations in the APC gene are associated with the colonic phenotype and several locations have been related to duodenal adenomas.*



Duodenal adenomas and carcinomas

As early as 1904, duodenal adenomas were reported by Funkestein [17] and the first published report of an FAP-related duodenal carcinoma appeared in 1935 [18]. Duodenal adenomas are observed in 50-90% of patients with FAP [19-21]. Duodenal adenomatosis can be graded according to Spigelman stage, which is a semi-quantitive scoring system, expressing the severity of duodenal polyposis according to number, size, histology and degree of dysplasia of the duodenal lesions (Table 1) [22].

Table 1: *Spigelman stage represents the severity in duodenal polyposis.*

Category	Points		
	1	2	3
Number of adenomas	1-4	5-20	> 20
Size of adenomas (mm)	1-4	5-10	> 20
Histology	Tubular	Tubulovillous	Villous
Degree of dysplasia	Mild	Moderate	Severe

Within each category, 1 to 3 points can be scored, and the sum of the score in the 4 categories results in the Spigelman stage. Stage 0, 0 points; Stage I, 1-4 points; Stage II, 5-6 points; Stage III, 7-8 points; Stage IV, 9-12 points.

The Spigelman stage increases with age, probably due to the increasing number and size of adenomas [23]. The duodenal adenomas are most often located around the ampulla of Vater. As a consequence of the localisation of these adenomas, the risk of pancreatitis seems higher in patients with FAP [24]. Furthermore, the clustering of the duodenal adenomas around the ampulla of Vater suggests that bile plays a role in the formation and/or progression of these adenomas.

The progression of duodenal adenomatosis is slow in patients with early disease [23, 25-27]. In two large studies, no malignant progression was present after 10 years in patients with Spigelman stage 0 or I duodenal adenomatosis [23, 26]. The cumulative incidence of malignancy in patients with Spigelman IV is estimated at between 7% [23] and 36% [26]. In patients surviving to the age of 75,

between 20-52% are likely to have developed Spigelman IV duodenal adenomatosis with an estimated cumulative incidence of duodenal carcinoma between 4 and 10% [23, 28, 29]. In general, duodenal carcinomas will develop in only 2-5% of the patients with FAP [19, 23, 26, 30] and their postoperative outcome is poor [31]. Considering the natural course of duodenal adenomatosis in patients with FAP, strategies for each Spigelman stage are summarised in Table 2 [32].

Table 2: *Recommended strategies in the managment of duodenal adenomatosis in patients with FAP, according to their Spigelman stage.*

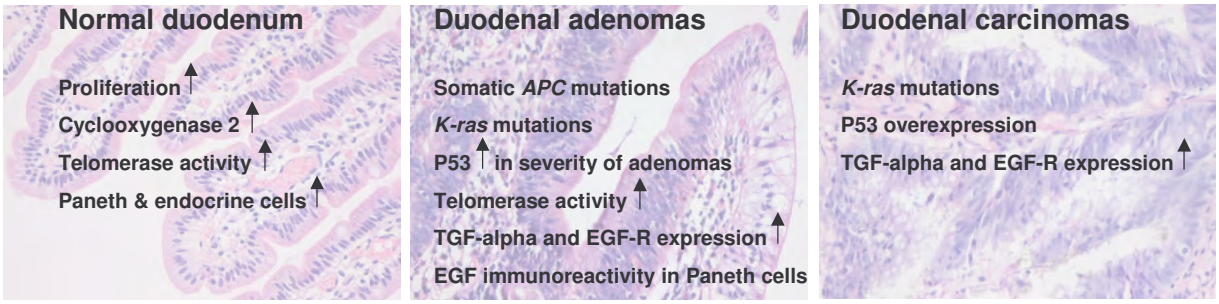
Spigelman Stage	Strategy
0	Side-viewing endoscopy including biopsy at intervals of 5 years.
I	Side-viewing endoscopy including biopsy at intervals of 5 years.
II	Side-viewing endoscopy including biopsy at intervals of 2-3 years.
III	Side-viewing endoscopy including biopsies at intervals of 1-2 years, chemoprevention with celecoxib and possibly endoscopic therapy.
IV	Pre-operative imaging, referral for consideration for prophylactic pylorus preserving pancreaticoduodenectomy, continued chemoprevention and surveillance and endoscopic resection in inoperable cases.

Developmental aspects of duodenal adenomas and carcinomas

In comparison to colorectal and gastric cancer, little is known about the carcinogenesis of the small intestine. In the small intestine, the most frequent site for the development of adenocarcinomas is the duodenum (± 50%), followed by the jejunum (± 25%) and the ileum (± 13%), whereas the localisation of the rest of the tumours is not specified [33-35]. The duodenum is also the main site for malignant extra-colonic manifestations in patients with FAP. Thus far, only a few reports have been published with regard to the

underlying mechanism of the development of adenomas and carcinomas in the duodenum of patients with FAP. Figure 2 summarises the publications describing abnormalities and possible mechanisms leading to the development of adenomas and carcinomas in the duodenum of patients with FAP.

Figure 2: *Abnormalities observed in the normal duodenum, duodenal adenomas or duodenal carcinomas in patients with FAP.*



In the normal duodenal mucosa of patients with FAP, several cellular abnormalities have been described; higher proliferation index [36, 37], increased expression of cyclooxgenase-2 (COX-2) [38], telomerase activity (43%) [39], increased number of Paneth and endocrine cells in the crypts [40]. In addition, in this thesis loss of extracellular E-cadherin is also described [41]. In duodenal adenomas of patients with FAP, somatic *APC* mutations are observed in 5%, 22% and 67% of the adenomas [42, 43]. *K-ras* mutations have been observed in 7% and 33% of the duodenal adenomas of patients with FAP [42, 44]. Telomerase activity (63%) [39] was also observed in the adenoma stage, as was overexpression of p53 [45]. In this thesis both loss of extracellular E-cadherin and SMAD4 was demonstrated in duodenal adenomas [41]. In addition, expression of transforming growth factor-alpha (TGF-alpha, 100%) [46] and epidermal growth factor receptor (EGF-R, 63%) expression [47] have also been described in duodenal adenomas of patients with FAP. In duodenal carcinomas, overexpression of p53 [45] was seen and *K-ras* mutations were detected in 12.5% of the ampullary carcinomas [44]. In analogy with the adenoma stage, TGF-alpha (100%) and EGF expression (67%) [46] was also

seen in the duodenal carcinomas. In Chapter 5 of this thesis, a comparison of chromosomal abnormalities and alterations in methylation of DNA is made between sporadic and FAP-related duodenal carcinomas. This comparison shows similarities as well as differences between duodenal tumours of hereditary and non-hereditary origin.

Bile acids

Since the duodenal adenomas in patients with FAP are mostly clustered around the ampulla of Vater, it has been suggested that bile acids may play a role in their initiation and/or progression. Primary bile acids are synthesised in the liver and are excreted into the gall bladder, from which they are released into the small intestine after stimulation of the gall bladder. Bile acids are then re-absorbed in the terminal ileum and only approximately 5% of the bile acid pool enters the large intestine. Bacteria in the large intestine are responsible for the transformation of primary bile acids into secondary bile acids. The secondary bile acids specifically are known tumour promoters in the gastrointestinal tract [48-50].

There is some evidence for an excess of DNA adducts (chemical modifications of DNA, that are implicated in the initiation of carcinogenesis) in the bile of patients with FAP [51-53]. The levels of DNA adducts seem to be pH dependent, indicating that a therapy increasing the duodenal pH may reduce damage of DNA caused by bile [54]. The findings that patients with FAP showing gastric adenomas have more severe reflux, may confirm the role of bile in adenomatosis [55]. Additionally, dysplasia is observed in the gall bladder of patients with FAP [56].

Biotransformation enzymes

The detoxification enzyme families, glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs), play an important role in the mucosal protection against potential toxins, mutagens or carcinogens. These enzyme families are responsible for the metabolism and excretion of many toxic, mutagenic or carcinogenic compounds, both of exogenous and endogenous

sources. UGTs catalyse the conjugation of mainly lipophilic compounds with glucuronic acid, while GSTs catalyse conjugation with glutathione of electrophiles and products of oxidative stress [57-59]. In general, the conjugates are more water soluble and less biologically active, and can be readily excreted. A significant inverse correlation between GST or UGT activity in the normal mucosa of the gastrointestinal tract and the corresponding tumour incidence has been observed [60], suggesting an important role for these enzymes in cancer prevention.

The human GSTs are a family of cytosolic enzymes, compried of four main classes: alpha, pi, mu and theta, each consisting of one or more isoforms [57]. The expression of the GSTs isoforms differs in the various tissues and large inter-individual differences in tissue levels have been reported [60-62]. The human UGTs are localised in the endoplasmic reticulum and are divided into the UGT1 and UGT2 subfamilies, consisting of 9 and 7 functional genes, respectively [63, 64]. Several UGT isoforms may be expressed in the duodenum as well as in the colon [65, 66], however the UGT enzyme activity in the duodenum was reported to be much higher compared to that of the colon [67].

In patients with FAP, a significantly lower GST activity was observed in the colonic mucosa as compared to healthy controls [68] . Furthermore, Spigelman *et al.* [69] reported lower GST mu levels in the blood of patients with FAP compared to controls. In addition, by phenotyping with caffeine [70] or genotyping [71], lower N-acetyltransferase (generally inactivating carcinogens) and higher cytochrome P-450 (generally activating carcinogens) enzyme activity was demonstrated in patients with FAP when compared to controls. Functional polymorphisms in the genes of detoxification enzymes may result in enzymes with reduced activity, or even in a complete absence of enzyme activity [72, 73]. Therefore, such functional polymorphisms could potentially modulate individual susceptibility for the development of duodenal adenomas and carcinomas in patients with FAP.

Chemoprevention

Several studies have been performed in regard to chemoprevention of duodenal adenomatosis in patients with FAP. Reduction of duodenal proliferation was observed after administration of the non-selective cyclooxygenase inhibitor sulindac [74]. However, Richard *et al.* [75] found no effect of sulindac on the reduction of ampullary adenomas, whereas Debinski *et al*. [76] only found an effect in small adenomas. By video endoscopic scoring, a statistically significant reduction of duodenal adenomatis was observed after 6 month's administration of 2x400 mg daily of the selective cyclooxygenase–2 inhibitor celecoxib [77]. In addition, celecoxib showed a significant reduction of colorectal adenomas, with concomittant change in relevant biomarkers in patients with FAP [78]**.** Gastric acid lowering therapy with ranitidine [79] or administration of calcium with calciferol [80] were not found to be effective in the reduction of duodenal adenomatosis. Recently, a reduction in the number and size of ileal adenomas was found after the combination of curcumin and quercetin [81]. Promising gene therapy, in which normal copies of the *APC* gene are supplied to the duodenum, has only been described *in vivo* [82].

Outline and objectives of this thesis

Duodenal carcinomas and desmoid tumours are now the leading causes of cancer–related mortality in patients with FAP. Therefore, the treatment of duodenal adenomas in patients with FAP is a clinical challenge. Since both the incidence and severity of the duodenal adenomatosis increase with age, and due to the colectomy the FAP population is aging, the problems with respect to the development of duodenal adenomas and malignancies will increase. So far, the treatment of duodenal adenomas in FAP has been mainly restricted to surgical intervention, with concomitant high morbidity and mortality, and therefore new therapies, such as chemoprevention, would be preferable. Although it is known that FAP is caused by mutations in the *APC* gene, the relation between the duodenal phenotype and the site of the *APC* mutations is not clear. Other factors could contribute to the variety seen in the age of onset, number of adenomas or severity of the duodenal adenomatosis. The studies described in the first part of this thesis focus on the role of biotransformation

enzymes in duodenal adenomatosis in patients with FAP. After the general introduction of **Chapter 1**, in **Chapter 2,** the detoxification capacity of UGT and GST in the duodenal mucosa of patients with FAP is compared with that of healthy controls. **Chapter 3** compares the genotype distribution of several important GST and UGT detoxification enzymes present in the duodenum in patients with FAP with the corresponding genotypes found in healthy controls. The following chapters of this thesis are focussed on the mechanisms underlying the development of duodenal adenomas and carcinomas in FAP. In **Chapter 4**, normal mucosa and adenoma tissues of the duodenum and colon of patients with FAP are compared with corresponding non-FAP samples for the distribution of E-cadherin, β-catenin and SMAD4. **Chapter 5** describes the chromosomal abnormalities and alterations in DNA methylation, as found in duodenal sporadic and FAP-related carcinomas. These findings are also compared to literature data for gastric and colorectal tumours. Finally, a pilot intervention study as described in **Chapter 6,** explores the possibility for the use of ursodeoxycholic acid as a future chemopreventive agent for duodenal adenomas/carcinomas in patients with FAP.

The main objectives of this thesis are to:

1. investigate factors that may influence the individual variety in duodenal adenomatosis in patients with FAP.
2. further elucidate the mechanisms underlying the development of duodenal adenomas and carcinomas in patients with FAP.
3. explore the possibility of chemoprevention of duodenal adenomas/ carcinomas in patients with FAP.

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Chapter 2

Detoxification enzymes in the duodenal mucosa of patients with familial adenomatous polyposis.

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Patients with familial adenomatous polyposis (FAP) are at risk of duodenal adenomas and carcinomas. Besides the germline mutations in the *APC* gene, which are responsible for the severity of the FAP phenotype, the disease may be modulated by other genes of low penetrance. The detoxifying enzymes glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) are involved in the mucosal protection against toxins and carcinogens. A low GST activity has been observed in the colon and blood of patients with FAP compared to that of controls. This suggests that these enzymes may be risk modulators in patients with FAP. The aim of this study is to compare the detoxification capacity of GST and UGT in duodenal mucosa of patients with FAP with that of healthy controls. Normal appearing duodenal biopsies were obtained from 18 patients with FAP and 18 controls. GST isoforms were quantified by immunoblotting. GST and UGT enzyme activities were measured spectrophotometrically and spectrofluorometrically respectively. No differences were observed in the GST isoform levels or in the GST and UGT enzyme activities, in the duodenal mucosa of patients with FAP compared to healthy duodenal mucosa. The detoxification enzymes GST and UGT studied here do not seem to be involved in the duodenal adenomatosis in patients with FAP.

Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder characterised by numerous adenomatous colorectal polyps that have a tendency to progress to adenocarcinoma. It is caused by germline mutations in the *Adenomatous Polyposis Coli* (*APC*) gene [1]. The treatment of choice is prophylactic colectomy since untreated FAP will inevitably lead to colorectal cancer at a relatively young age [2]. Patients with FAP are also at risk of developing extra-colonic manifestations, although the duodenum is the main site for these malignant manifestations [3,4]. The prevalence of the mainly peri-ampullary adenomas varies from 50% to greater than 90% [7-9]. The cumulative incidence of duodenal adenomatosis at the age of 70 years is 90%, and 52% of these adenomas are graded as Spigelman stage IV [10], which is associated with a high risk of a malignant course of the adenomas [11-13]. The prevalence of duodenal carcinoma in patients with FAP is 2-5% [4,5,8,10,12] and compared to the general population, the relative risk of duodenal adenocarcinoma is exceptionally high [3]. However, since the FAP populations investigated so far are relatively young, the prevalence rates will increase further [12]. Since the incidence and severity of the duodenal adenomatosis increases with age regular endoscopic surveillance is recommended. The current treatment of choice for patients with Spigelman stage IV adenomas is a pylorus-preserving pancreaticoduodenectomy [10]. However the choice between continued surveillance and this procedure, which is associated with considerably morbidity and mortality, is delicately balanced [14]. Therefore chemoprevention would be a much more preferable treatment [15], mainly since celecoxib, a selective cyclooxygenase-2 inhibitor, showed a beneficial effect in the prevention or even regression of duodenal adenomatosis [16].

Although mutations in the *APC* gene are the main cause of FAP, modifier genes of low penetrance are likely to contribute to the FAP phenotype, and carcinogen metabolising enzymes may be good candidates [17]. The detoxifying enzymes glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) are involved in the detoxification of carcinogens and have a pivotal function in mucosal protection against carcinogens. The human GSTs are a family of enzymes, compried of four

main classes: alpha, pi, mu and theta, each consisting of one or more isoforms [18]. GSTs catalyse the conjugation of a wide variety of toxic or carcinogenic electrophiles to the tripeptide glutathione. The majority of these GST substrates are xenobiotics or products of oxidative stress. The conjugation of electrophiles with the sulfhydryl group of glutathione generally results in more water soluble and less toxic molecules, which are subsequently excreted via bile or urine, and thus GSTs are almost exclusively involved in detoxification [18,19]. The expression of the GSTs isoforms differs in the various tissues and large inter-individual differences in tissue levels have also been reported [20-23]. A significant inverse correlation between GST activity in normal mucosa along the gastrointestinal tract and the tumour incidence at these sites has been observed [22], suggesting an important role for GST in cancer prevention.

The UGTs are endoplasmic reticulum resident enzymes, which catalyse the transfer of glucuronic acid from the donor UDP-glucuronic acid to a wide range of potentially hazardous molecules. This glucuronidation is responsible for the transformation of lipophilic substrates into more easily renal or biliary excreted, hydrophilic glucuronides, which in general are less toxic compared to the parent compound [24]. Human UGTs are divided into the UGT1 and UGT2 subfamilies, consisting of 9 and 7 functional genes respectively [25,26]. Several UGT isoforms may be expressed in the duodenum as well as in the colon [27,28], however the UGT enzyme activity in the duodenum was reported to be much higher compared to that of the colon [21,27,29].

Recently, we noticed a significantly lower GST activity in the colonic mucosa of patients with FAP compared to healthy controls [30], whereas lower GST mu levels were measured in the blood of patients with FAP compared to controls by Spigelman *et al*. [31]. In addition, via caffeine phenotyping [32] or genotyping [33], lower N-acetyltransferase (NAT; generally inactivating carcinogens) and higher cytochrome P-450 (generally activating carcinogens) activity was demonstrated in patients with FAP compared to controls. Lower levels of NAT, GST or UGT activity in the intestine may enhance the risk of developing intestinal carcinoma [21]. Therefore, it is suggested that reduced detoxification activity may to some extent modulate the risk of (extra)-colonic adenomas or

carcinomas in patients with FAP. The aim of this study is to compare the detoxification capacity of GST and UGT in the duodenal mucosa of patients with FAP with that of healthy controls.

Material & Methods

Patients and controls

Duodenal biopsies were obtained from 18 patients (average age 49 ± 15 yrs, 10 males/ 8 females) with FAP, who underwent routine duodenoscopy at the Radboud University Nijmegen Medical Centre, the Netherlands. The specimens were taken from normal appearing mucosa. Severity of duodenal adenomatosis was classified according to Spigelman stage [34]. Spigelman 0, I, II, III and IV was found in 4, 4, 4, 5 and 1 patients respectively. In the period from 1957-1997, 17 patients underwent colectomy. Control duodenal biopsies were obtained from 18 subjects (average age 50 ± 13 yrs, 8 males / 10 females) who underwent duodenoscopy for suspected coeliac disease, however no abnormalities were found by the pathologist. Informed consent was given by the individuals participating in this study.

Tissue samples

Immediately after endoscopy, three biopsy specimens were frozen in liquid nitrogen and stored at – 80° C until use. The biopsy specimens were weighed and homogenised on ice in 5 parts homogenising buffer (100 mM Tris/HCL, 1mM EDTA, pH 7.4). A part of the homogenates were immediately frozen in liquid nitrogen and stored at – 80^0 C for the UGT activity assay, and the rest of the homogenates was centrifuged at 150,000 g at 4° C for 1 hour (cytosolic fractions) for the GST assays. Immediately after centrifugation, the cytosolic fractions were frozen in liquid nitrogen and stored in small aliquots at – 80° C until further analysis.

Glutathione S-transferase assays

Protein concentrations were determined in duplicate by the method of Lowry *et al.* [35] using bovine serum albumin as a standard. The cytosolic glutathione S-

transferase activity was assayed in duplicate with 1-chloro, 2,4-dinitrobenzene as substrate by using the method of Habig *et al.* [36]. Concentrations of the GST alpha, mu, pi and theta isoforms were determined by immunoblots previously as described [22,37,38]. In short, cytosolic fractions were subjected to sodium dodecylsulfate-polyacrylamide 13% (w/v) gel electrophoresis (SDS-PAGE). Subsequently, proteins were electroblotted to nitrocellulose membranes which were then blocked with 1% gelatine in PBS-buffer also containing 0.05% (v/v) Tween-20 (PBS-T) for 15 min. Next, the membranes were incubated overnight with monoclonal antibodies (dilution 1:5000 in PBS-T/ gelatine) against human GST class alpha (A1 + A2) [37], pi (P1) [39], mu (M1 + M2) [40] or theta (T1) [41]. After washing and incubating with the secondary antibody, peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts Glostrup, Denmark), immunoreactive proteins were visualized with 0.1% (w/v) 3,3'-diaminobenzidine, 5 mM imidazole, 2 mM cobalt chloride· $6H_2O$ and 16 µl 30% H_2O_2 in PBS. Intensity of the staining was measured using a laser densitometer (Ultrascan XL; LKB, Bromma, Sweden). Known amounts of the corresponding GST isoforms, which were run in parallel with the samples, were used as standards for the quantification of the GST isoforms.

UDP- glucuronosyltransferase enzyme activity measurement

UGT activity was assayed with 4-methylumbelliferone (4-MUB) as substrate [42]. Duodenum homogenates were preincubated for 2 mins at 37˚ C in the presence of 10 mM $MgCl_2$, 1 mM saccharic aicd-1,4-lactone and 0.1 mM 4-MUB in a final volume of 110 µl Tris/HCl (20 mM), pH 7.4. The substrate 4-MUB was dissolved in ethanol (50 mM) and diluted with assay medium just before use. Subsequently, UDP-glucuronic acid (UDPGA; Sigma, Zwijndrecht, the Netherlands) was added (concentration during incubation: 4 mM) and the samples were incubated for 30 mins at 37˚ C. After the addition of 1 ml 0.5 M glycine/ NaOH, pH 10.35, the conjugation reaction was ended. Next the samples were centrifuged at 10,000 g for 10 min and 4-MUB was determined fluorometrically (excitation 375 nm, emission 450 nm).

Statistical analysis

The Wilcoxon-Mann-Whitney test was used to compare enzyme activities and GST isoenzyme levels of patients and controls. The Kruskal-Wallis test was performed to examine the relation between the enzyme activities, GST isoenzyme levels and the categorical variable Spigelman classification. $P < 0.05$ was considered to be statistically significant (SPSS 11.0 for Windows, 2001).

Results

Duodenal biopsies from 18 patients with FAP and 18 patients with normal duodenal mucosa (controls) were analysed in this study. Patients with FAP and controls were matched with respect to age and gender. Data on the levels of glutathione S-transferase alpha (A1 + A2), pi (P1), mu (M1) and theta (T1), the GST and UGT enzyme activities in the duodenal mucosa of patients with FAP and healthy controls is presented in Table 1. Seven patients with FAP and 7 controls were of the GSTM1 null genotype and showed no expression of the GSTM1 protein, whereas 6 patients with FAP and 2 controls were of the GSTT1 null genotype.

Table 1: *Glutathione S-transferase (GST) isoenzyme content, GST and UDP-glucuronosyltransferase (UGT) enzyme activity in duodenal mucosa of patients with FAP and controls*

	FAP n = 18	**Controls** n = 18
GSTA1 + A2 in ng / mg protein	7138 (5830 - 7917)	6166 (5128-7975)
GSTP1 in ng / mg protein	4617 (2644-5830)	4463 (3175-5294)
GSTM1 in ng / mg protein	325 (0-429)	344 (0-426)
GSTT1 in ng / mg protein	2376 (1937-5320)	2383 (1675-4567)
GST enzyme activity in nmol min^{-1} mg^{-1} protein	619 (586-689)	576 (531-697)
UGT enzyme activity in nmol min^{-1} mg^{-1} protein	0.45 (0.27-0.59)	0.47 (0.28-0.65)

Data are presented as medians (25^{th} -75^{th} percentiles).
GSTM1 negative: 7 patients and 7 controls
GSTT1 negative: 6 patients and 2 controls

GST A1 + A2 levels were highest (7138 vs. 6166 ng/mg protein p = 0.372), followed by GSTP1 (4617 vs. 4463 ng/mg protein p = 0.501), GSTT1 (2376 vs. 2383 ng/mg protein p = 0.377) and GSTM1 (325 vs. 344 ng/mg protein p = 0.748) in duodenum of patients with FAP compared to controls. No differences were observed in the GST levels in the duodenal mucosa of patients with FAP compared to healthy duodenal mucosa. Furthermore no differences in the enzyme activity of GST (p = 0.606) or UGT (p = 0.935) in the duodenal mucosa of the patients with FAP compared to values in control mucosa could be noticed.

The Spigelman stage is a measure for the severity of the duodenal adenomatosis in patients with FAP. In this study no relation was found between the Spigelman stages and the GST and UGT enzyme activities (p = 0.353 and p = 0.700 respectively). Nor did the GST isoform levels show a relation with the Spigelman stages.

Discussion

Patients with the autosomal dominant disease FAP develop hundreds of adenomas in their colon. Without prophylactic colectomy, these will inevitable lead to colon cancer at a relatively young age. Nowadays, with an increased life expectancy for patients with FAP because of this prophylactic colectomy, the problems of the extra-colonic manifestations in these patients become apparent [8,10-12]. Adenomas are also formed in the upper gastrointestinal tract, most notably in the duodenum around the ampulla. [5,6]. This process, although mainly driven by *APC* mutations, may be modulated to some extent by other factors, such as levels and functioning of detoxification enzymes.

The GST and UGT detoxification enzymes are involved in the mucosal protection against carcinogens. GSTs and UGTs are widely distributed along the gastrointestinal tract and both enzyme systems significantly contribute to the prevention of carcinogenesis at these sites [21,22,23]. A lower detoxification capacity, however, could contribute to an enhanced cancer risk. For example,

a low UGT and GST enzyme activity is present in the colon compared to the small intestine, whereas the cancer incidence in the colon is much higher compared to that in the small intestine [21,22,27,29]. Furthermore, a significantly lower GST enzyme activity was observed in the colonic mucosa and blood of patients with FAP compared to corresponding values in controls [30,31]. However, direct measurement of detoxification enzyme values at sites other than the colon [30] in the gastrointestinal tract of patients with FAP has never been performed and therefore we investigated UGTs and GSTs in the duodenum, a site where adenomas and carcinomas also develop.

This study, however, reveals no differences between the levels of GST isoforms and GST and UGT enzyme activity in the duodenal mucosa of patients with FAP compared to normal duodenal mucosa of age and sex matched controls. Thus, in contrast to our earlier observation in the colon, a lowered detoxification capacity does not seem to be present in the duodenal mucosa of patients with FAP. The much higher detoxification capacity in the upper-gastrointestinal tract compared to that of the colon may play a role in the differences in the prevalence of adenomas and carcinomas seen at these sites in patients with FAP. The prevalence of duodenal adenomas and carcinomas is 50-90% and 2-5% [4,5,8,10,12] compared to corresponding values of 100% and 90% respectively in the colon [43].

A lower detoxification capacity is associated with a higher risk of carcinogenesis [20,21,22]. Genetic polymorphisms in genes encoding for GST and UGT isoforms can lead to an absence or decrease in enzyme activity [18,44]. Crabtree *et al*. reported an association between N-acetyltransferase (NAT) polymorphisms and more severe disease in FAP, although no associations with other polymorphisms leading to reduced enzyme activity, such as GST M1 or GSTT1 null polymorphisms, were found [17]. The latter is in accordance with the results of Lamberti *et al*., who found no association between GSTM1 null genotype and age at first diagnosis of colorectal adenomas or extra-colonic manifestations [45]. Although there is some evidence of a modulating role of genes of low penetrance in the severity and distribution of adenomas and carcinoma in the duodenum of patients with FAP, direct enzyme activity

measurements in the target organ have not so far been performed. No evidence was found however that lower levels or enzyme activity of potential modifying enzymes were present in the duodenum of patients with FAP.

Since there are mainly peri-ampullary adenomas present in the duodenum of patients with FAP, it seems that bile may be a strong tumour promoter, or that otherwise the peri-ampullary mucosa may be more sensitive towards adenomatosis compared to the more distant mucosa. This was difficult to test however, as normal peri-ampullary mucosa of patients with FAP could not be obtained.

In summary, the detoxification capacity of GSTs and UGTs in the duodenum is not lower in patients with FAP compared to healthy duodenal mucosa. Therefore, these detoxification enzymes do not seem to be involved in the duodenal adenomatosis in FAP.

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Chapter 3

Polymorphisms in detoxification enzymes are not involved in duodenal adenomatosis in patients with familial adenomatous polyposis

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Patients with familial adenomatous polyposis (FAP) are at high risk of duodenal adenomas and carcinomas. Besides germline mutations in the *APC* gene, the age of onset and the number of duodenal adenomas may be modulated to some extent by additional factors, such as the detoxification enzymes glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs), which are involved in the mucosal protection against toxins and carcinogens. This study compares the genotype distributions of duodenal isoforms of UGTs and GSTs in patients with FAP and controls. Genotyping of 85 patients with FAP and 218 healthy controls was performed for polymorphisms in the detoxification enzymes UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A10, UGT2B4, UGT2B7, UGT2B15, GSTA1, GSTP1, GSTM1 and GSTT1.

The variant genotypes of *UGT1A3* were found significantly less common in patients with FAP (OR 0.39, 95% CI 0.22-0.67) compared with controls, whereas no associations were found between FAP and the other polymorphic genes. The polymorphisms investigated here had no predictive value for the severity of the duodenal adenomatosis in the patients with FAP. The variant genotype of *UGT1A3* is less present in patients with FAP, however this variant genotype does not modulate the severity of duodenal adenomatosis.

Introduction

FAP is an autosomal dominant disorder caused by germline mutations in the tumour suppressor gene a*denomatous polyposis coli* (*APC*) [1]. Patients with FAP develop hundreds to thousands of colorectal adenomas which without prophylactic colectomy will inevitably lead to colorectal cancer at a relatively young age [2]. Nowadays, with increased survival due to prophylactic colectomy, problems of extra-colonic manifestations become apparent in these patients [3]. The prevalence of the mainly peri-ampullary adenomas in patients with FAP varies from 50-90% [4-6] and the prevalence of duodenal carcinoma is 2-5% [3, 7]. Together with desmoid tumours, duodenal carcinomas are now the leading causes of cancer-related mortality in patients with FAP [8].

The severity of the colonic phenotype can be associated with the site of the *APC* mutations. In contrast, the development of duodenal adenomas can only be partly explained by the location of the *APC* mutations [9]. Furthermore, there is a large variety in the age of onset as well as in the number of duodenal adenomas in patients with FAP. This suggests that, in addition to the *APC* mutations, other genes of low penetrance could play a modulating role in the inter-individual differences in the development of duodenal adenomas and carcinomas. Genes of biotransformation enzymes could be good candidate genes [10]. The detoxification enzyme families glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) especially, which play an important role in the mucosal protection against potential mutagens and carcinogens. These enzyme families are responsible for the metabolism and excretion of many toxic, mutagenic or carcinogenic compounds, both of exogenous and endogenous sources. UGTs catalyse the conjugation of mainly lipophilic compounds with glucuronic acid, while GSTs catalyse conjugation with glutathione of electrophiles and products of oxidative stress [11-13]. After conjugation, the compounds are generally more water soluble and less biologically active, and can be readily excreted. A significant inverse correlation has been observed between GST or UGT activity in the normal mucosa of the gastrointestinal tract and the corresponding tumour incidence [14], suggesting an important role for these enzymes in cancer prevention. Several isoforms of the UGT and GST detoxification enzyme families may be

expressed in the duodenal mucosa (GSTM1, GSTP1, GSTT1, GSTA1, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A10, UGT2B4, UGT2B7 and UGT2B15) [15-19]. Functional polymorphisms in the genes of these detoxification enzymes may result in reduced enzyme activity, or even in a complete absence of enzyme activity [20, 21]. Therefore, such functional polymorphisms could result in deficient duodenal detoxification, leading to increased levels of harmful compounds, and could thus potentially modulate individual susceptibility to the development of duodenal adenomas and carcinomas in patients with FAP.

Until now, there have been several reports on biotransformation enzymes in patients with FAP. Spigelman *et al*. [22] reported a lower activity of GSTM in the peripheral blood of patients with FAP compared with that of controls. However, in duodenal mucosa of patients with FAP we were not able to demonstrate lower UGT and GST enzyme activity compared to controls [23]. By caffeine phenotyping, lower N-acetyltransferase (NAT; generally inactivating carcinogens) and higher cytochrome P4501A2 (generally activating carcinogens) activity was demonstrated in patients with FAP compared to controls [23]. Furthermore, slow acetylation phenotypes were shown to increase the risk of extra-colonic manifestations in patients with FAP [24] . Genetic polymorphisms in *NAT*, but not in *GSTT* or *GSTM*, were shown to modulate the severity of disease in patients with FAP [10, 25] .

The GST and UGT enzyme activity measurements in duodenal mucosa of patients with FAP and controls, performed by us in an earlier study [15] cover only part of the GST and UGT isoenzymes present in the duodenum. In addition, activity measurements were performed only on a limited number of patients. Therefore in the current study, the genotypes of the most important duodenal isoforms of the UGT and GST family were determined in a large number of patients with FAP and compared with those of controls.

Materials & Methods

Patients and controls

A total of 85 patients with FAP (average age 42 ± 16 yrs, 38 males/ 47 females) were included in this study; 59 patients were recruited at the Department of Gastroenterology, Radboud University Nijmegen Medical Centre, and 26 patients were recruited at the Department of Gastroenterology and Hepatology, Academic Medical Centre, Amsterdam, the Netherlands. All patients with FAP were unrelated, meaning that only one patient per family name was included. Fifty-nine patients with FAP were diagnosed based on the presence of *APC* mutations and 26 of the patients on the specific clinical characteristics; namely the presence of hundreds of adenomas in their colon. Age and gender matched healthy subjects (218 in total, average age 42 ± 16 yrs, 103 males/ 115 females) served as controls and were recruited by an advertisement in a local paper in the area of Nijmegen. All subjects were of Caucasian origin. This study was approved by the local medical ethical review committee.

Spigelman stage

Of 50 patients with FAP (59%), age as well as the Spigelman stage was registered at first duodenoscopy. Spigelman's stage is a semi-quantitive scoring system, expressing the severity of duodenal polyposis according to number, size, histology and degree of dysplasia of duodenal lesions, as outlined in Table 1 [26].

Table 1: *Spigelman stage represents the severity in duodenal polyposis.*

Category	Points		
	1	2	3
Number of adenomas	1-4	5-20	> 20
Size of adenomas (mm)	1-4	5-10	> 20
Histology	Tubular	Tubulovillous	Villous
Degree of dysplasia	Mild	Moderate	Severe

Within each category, 1 to 3 points can be scored, and the sum of the score in the 4 categories results in the Spigelman stage. Stage 0, 0 points; Stage I, 1-4 points; Stage II, 5-6 points; Stage III, 7-8 points; Stage IV, 9-12 points.

DNA isolation

The Puregene DNA purification kit (Gentra Systems, Minneapolis, MN) was used to isolate DNA from whole blood of 64 patients with FAP and 218 healthy controls. The isolation was performed according to the instructions of the manufacturer. From 21 patients with FAP, no blood samples were available and here DNA was isolated from resected colonic tissue by using the phenol-chloroform-isoamylalcohol extraction method according to Maniatis *et al*. [27].

Genotyping

UGT1A1. The number of TA repeats in the promoter region of the *UGT1A1* gene was analysed using the polymerase chain reaction (PCR) conditions and primers (Table 2) according to Monaghan *et al*. [28]. After confirmation of the amplification by agarose electrophoresis, the fragments were subjected to 10% polyacrylamide gels (19:1 acrylamide / bisacrylamide) in Tris – Borate - EDTA buffer. Gels (20 x 20 x 0.075 cm) were run at 400V for 3 hrs and stained with ethidium bromide for 30 mins [29]. Fragments of 98 bp indicate the *UGT1A1*1* allele, which contains six TA repeats, and fragments of 100 bp indicate the *UGT1A1*28* allele with seven TA repeats.

UGT1A3 and UGT1A4. Polymorphic variants of the *UGT1A3* and *UGT1A4* genes were identified by PCR-restricted fragment length polymorphism (RFLP) methods developed in our laboratory. To detect the variations at UGT1A3 codons 11 and 47, the forward and reverse primers, as shown in Table 2, were used in the presence of 1.5 mM $MgCl_2$. The PCR conditions were: 5 mins at 95°C, then 40 cycles of 30 secs at 95°C, 30 secs at 50°C, 1 min at 72°C and finally an elongation step of 7 mins at 72°C. A 427 bp product was amplified and aliquots of 5 µl of the PCR mixture were digested for at least 1 h at 37 ˚ C with the appropriate restriction enzyme, followed by electrophoresis on 3% agarose gel, containing ethidium bromide. Electrophoresis patterns of DNA fragments after digestion with SstII for codon 11 are: a 427 bp product (homozygous common genotype), 427 + 319 + 108 bp products (heterozygous genotype) and 319 + 108 bp products (homozygous variant genotype). For codon 47, digestion occured with the restriction enzyme StuI, resulting in an electrophoresis pattern of a 427 bp

product (homozygous most common), 427 + 217 + 210 bp products (heterozygous genotype) and 217 + 210 bp products (homozygous variant genotype).

Table 2: *Primers and restriction enzymes used for genotyping*

Genes	Primers/beacons (5'- 3'; F = forward, R = reverse) *	Restriction enzymes
UGT1A1	F : GTC ACG TGA CAC AGT CAA AC R : TTT GCT CCT GCC AGA GGT T	-
UGT1A3	F : AGG TAA TTA AGA TGA AGA AAG R : ACA TTG CCA TAC TTC TGA	W11R : *SstII* V47A : *StuI*
UGT1A4	F : GCC CAT AAC GAA AGG CAG T R : CAC ACA ACA CCT ATG AAG GG	P24T : *Hpy188III* L48V : *StuI*
UGT1A6	F : GGA AAA TAC CTA GGA GCC CTG TGA R : AGG AGC CAA ATG AGT GAG GGA G	T181A : *NSiI* R184S : *Fnu4HI*
UGT1A10	F : CTC TTT CCT ATG TCC CCA ATG R : CTG GAA AGA AAT CTG AAA TGC AAC AAA C	T240M : *NlaIII* L244I : *EcoRV*
UGT2B4	F : CCA AAT TAA CTT ACT TTC AAT GTT R : AGG CCC AGC AGG AAC CCA G Fam-CGC GAT AGC CCC TTG ATC GAG CAG TCA TCG CG-BHQ1 Hex-CGC GAT AGC CCC TTG AAA GAG CAG TCA TCG CG-BHQ1	-
UGT2B7	F : GTA TGG CTT ATT CGA AAC TC R : CAA ACA CTC TGA AAG AAG AC Fam-TTT CAG TTT CCA TAT CCA CTC TTA CC-BHQ1 Hex-TTT CAG TTT CCT CAT CCA CTC TTA CC-BHQ1	-
UGT2B15	F : TTG ACA TCT TCG GCT TCT AC R : CTG CCA GAA TGA CAT CAA AC Fam-CGC TGC CAT CTT TAA CTA AAA ATG ATT TGG AAG ATT CTC TGC AGC G-BHQ1 Hex-CGC TGC CAT CTT TAA CTA AAA ATT ATT TGG AAG ATT CTC TGC AGC G-BHQ1	
GSTA1	F : TGT TGA TTG TTT GCC TGA AAT T R : GTT AAA CGC TGT CAC CGT CC	*EarI*
GSTP1	F: GTA GTT TGC CCA AGG TCA AG R: AGC CAC CTG AGG GGT AAG	*Alw261*
GSTM1	F : CTC CTG ATT ATG ACA GAA GCC R : CTG GAT TGT AGC AGA TCA TGC	-
GSTT1	F : TTC CTT ACT GGT CCT CAC ATC TC R : TCA CCG GAT CAT GGC CAG CA	-
ß-Globin	F : CAA CTT CAT CCA CGT TCA CC R : GAA GAG CCA AGG ACA GGT AC	

The polymorphic variants of UGT1A4 codons 24 and 48 were detected using the forward and reverse primers, as shown in Table 2, with 2.0 mM $MgCl_2$. The PCR conditions were: 5 mins at 95°C, then 40 cycles of 30 secs at 95°C, 30 secs at 54°C, 1 min at 72°C and finally an elongation step of 7 mins at 72°C. Aliquots (5 µl) of the PCR product were digested with the restriction enzyme Hpy188III (codon 24) or StuI (codon 48) for at least 1 h at 37˚C. For codon 24, this digestion resulted in 513 + 54 bp products (homozygous most common), 513 + 266 + 247 + 54 bp products (heterozygous genotype), 266 + 247 + 54 bp products (homozygous variant genotype). The digestion with StuI leads to electrophoresis patterns of 319 + 248 bp products (homozygous most common), 567 + 319 + 248 bp products (heterozygous genotype) and a 567 bp product (homozygous variant genotype).

UGT1A6 and UGT1A10. The polymorphisms in the *UGT1A6 and UGT1A10* genes were studied using PCRs followed by RFLP analyses as previously described [30-32]. Table 2 shows the primers and restriction enzymes used in these studies.

UGT2B4, UGT2B7 and UGT2B15. A dual-colour allele-specific assay was used for genotyping the polymorphism at codon 458 of the *UGT2B4* gene. PCR was performed on the iCycler iQ Multicolour Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) using forward and reverse primers in the presence of the FAM-labelled wild-type beacon and the HEX-labelled mutant beacon (Sigma-Aldrich, Zwijndrecht, the Netherlands), as described in Table 2. The 25 microliter reaction mixture contained 200 ng of genomic DNA, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 4 mM $MgCl_2$, 0.25 mM dNTPs, 5 pmol of each primer, 200 nM of each beacon and 2.5 U Taq-DNA-polymerase. The PCR conditions were 3 mins at 95°C, then 40 cycles of 30 secs at 95°C, 30 secs at 56°C and 30 secs at 72°C. Fluorescent signals were measured at 56°C. Genotypes were assigned using the iCycler iQ Optical System Software version 3.1. At each PCR run (in 96 wells plates), sterile H_2O rather than genomic DNA, was added to several wells as negative controls for amplification. For the genotyping of the exon 1 polymorphism at codon 268 of the *UGT2B7* gene, a similar method was used as described above for

UGT2B4. For *UGT2B7*, the PCR conditions were 3 mins at 95°C, then 40 cycles of 30 secs at 95°C, 30 secs at 57°C and 30 secs at 72°C. Table 2 describes primers and probes (Sigma-Aldrich) used for *UGT2B7*. For the genotyping of the exon 1 polymorphism at codon 85 of the *UGT2B15* gene, the same method was used as described above for *UGT2B4* but with a magnesium concentration of 3 mM . For this polymorphism, the PCR conditions were 3 mins at 95°C, then 40 cycles of 30 secs at 95°C, 30 secs at 60°C and 30 s at 72°C.

GSTM1 and GSTT1. The *GSTM1* polymorphism was determined by PCR according to the method of Brockmöller *et al*. [33]. The *β-globin* gene was used as an internal positive control and sterile H_2O, as substitute for genomic DNA, served as a negative control. PCR primers are given in Table 2. PCR amplification was followed by 1% agarose gel electrophoresis of the PCR products. The presence of a 650 bp product represents at least one functional *GSTM1* allele, whereas absence of an amplification product is consistent with the null genotype. The detection procedure of the *GSTT1* null polymorphism based on the method of Pemble *et al*. [34] is similar to that of GSTM1. Here the visualization of a 480 bp product indicates the presence of at least one functional *GSTT1* allele.

GSTA1 and *GSTP1.* The polymorphisms in the *GSTA1 and GSTP1* genes were studied by using PCR, followed by RFLP analyses exactly as described before [35,36]. Table 2 shows the primers and restriction enzymes used for these polymorphisms. All genotypes investigated in this study are summarised in Table 3.

Table 3: *Summary of the polymorphic variants of the detoxification enzymes.*

Nomenclature polymorphic genes	Nucleotide or amino acid changes *
UGT1A1*1	$(TA)_6TAA$
UGT1A1*28	$(\mathbf{TA})_7TAA$
UGT1A3*1	$W^{11}V^{47}$
UGT1A3*2	$\mathbf{R}^{11}\mathbf{A}^{47}$
UGT1A3*3	$\mathbf{R}^{11}V^{47}$
UGT1A3*6	$W^{11}\mathbf{A}^{47}$
UGT1A4*1	$P^{24}L^{48}$
UGT1A4*2	$\mathbf{T}^{24}L^{48}$
UGT1A4*3	$P^{24}\mathbf{V}^{48}$
UGT1A6*1	$T^{181}R^{184}$
UGT1A6*2	$\mathbf{A}^{181}\mathbf{S}^{184}$
UGT1A6*3	$T^{181}\mathbf{S}^{184}$
UGT1A10*1	$T^{240}L^{244}$
UGT1A10*3	$\mathbf{M}^{240}L^{244}$
UGT1A10*4	$T^{240}\mathbf{I}^{244}$
UGT2B4*1	D^{458}
UGT2B4*2	$\mathbf{E}^{458}$
UGT2B7*1	H^{268}
UGT2B7*2	$\mathbf{Y}^{268}$
UGT2B15*1	D^{85}
UGT2B15*2	$\mathbf{Y}^{85}$
GSTA1*A	-69C
GSTA1*B	-69**T**
GSTP1 Ile 105	I^{105}
GSTP1 Val 105	$\mathbf{V}^{105}$
GSTM1+	At least one functioning allele
GSTM1null	Deletion in both alleles
GSTT1+	At least one functioning allele
GSTT1 null	Deletion in both alleles

* Bold characters indicate nucleotide or amino acid changes as compared to the most common allele.

Statistical analysis

The differences between characteristics of patients with FAP and controls were analysed with the Student's t-test. All genotypes investigated among controls and patients were tested as to whether they were distributed according to the Hardy-Weinberg equilibrium. Furthermore, the chi-square was used to test for differences in the distribution of genotypes between the two study groups, or to estimate differences in allele frequencies. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated for genotypes associated with predicted normal enzyme activity versus genotypes with predicted altered enzyme activities (variant genotypes). The predictive value of the various polymorphisms for the Spigelman stage at the first duodenoscopy of the patients with FAP was determined by logistic regression. In total, five different genetic polymorphisms of the UGT1A family were analysed. Because genetic polymorphisms were only detected in four of these UGT1A genes that are all derived from one combined gene locus, we corrected for multiple testing with Bonferroni here, implying that a p-value of 0.0125 was considered to be statistically significant. In all other cases, a $P < 0.05$ was considered to be statistically significant (SPSS 12.0.1 for Windows 2003, SPSS Inc, Chicago, USA).

Results

Characteristics of patients and controls

Table 4 lists the characteristics of the patients with FAP and controls. In 34% of the patients with FAP, a Spigelman stage 0 was observed at first duodenoscopy. Only 4% showed Spigelman stage I compared with 24%, 22% and 16% for the stages II, III and IV, respectively.

Table 4: *Characteristics of patients with FAP and controls.*

Characteristics	Patients with FAP	Controls
Number	85	218
Age (years; mean ± SD)	42 ± 16	42 ± 16
Gender:		
Female	47 (55.3)	115 (52.8)
Male	38 (44.7)	103 (47.2)
Spigelman stage at first duodenoscopy [A]		
Stage 0	17 (34.0)	
Stage I	2 (4.0)	
Stage II	12 (24.0)	
Stage III	11 (22.0)	
Stage IV	8 (16.0)	
Age at first duodenoscopy (years; mean ± SD)		
Stage 0	39.6 ± 11.8	
Stage I	63.0	
Stage II	43.0 ± 12.2	
Stage III	38.3 ± 10.0	
Stage IV	48.5 ± 10.2	

[A] For 35 patients with FAP, no information on Spigelman stage at first duodenoscopy was available. Percentages are given in parenthesis

Genotyping

Genotype distributions of the UGT and GST biotransformation enzymes investigated here are summarised in Table 5, and corresponding allele frequencies are shown in Table 6. All polymorphisms tested here were distributed according to the Hardy-Weinberg criteria, except for two genotypes, which did not fit these criteria: the *UGT1A4* polymorphism in the control group and the *UGT2B7* polymorphism in the patient group. Chi-square analyses revealed no significant differences in the investigated polymorphisms between patients with FAP and controls. The difference observed between patients with FAP and the control group for the *UGT1A3* gene ($p = 0.013$), lost significance after the Bonferroni correction for multiple testing ($p < 0.0125$).

Calculation of the odds ratio of genotypes associated with predicted normal enzyme activity versus genotypes associated with predicted altered enzyme activities (variant genotypes) showed that the variant genotypes of *UGT1A3* are significantly less common in patients with FAP (OR 0.39, 95% CI 0.22-0.67). The observed lower frequency of the variant genotypes of *UGT1A1* in patients with FAP ($p = 0.021$) was no longer significant after the Bonferroni correction for multiple testing ($p < 0.0125$).

Chi-square analyses revealed no significant differences in the presence of variant alleles between patients with FAP and controls. When combining alleles that may provide normal enzyme activities versus alleles that may yield altered enzymes activities, a significantly lower number of the variant *UGT1A3* alleles were observed in the patients with FAP (OR 0.60, 95% CI 0.41-0.88).

Spigelman stage

No associations between genotype patterns and the Spigelman stages were observed in patients with FAP. Furthermore, the polymorphisms investigated here had no predictive value for the Spigelman stage at first duodenoscopy in patients with FAP.

Table 5: *Distribution of UGT and GST genotypes in patients with FAP and controls.*

Genes	Genotypes	Patients with FAP (%) [A]	Controls (%) [A]	OR (95% CI) [B]
UGT1A1	*1*1 *1*28 *28*28	49/85 (57.7) 28 (32.9) 8 (9.4)	94/216 (43.5) 98 (45.4) 24 (11.1)	0.57 (0.34-0.94)
UGT1A3	*1*1 *1*2 *2*2 *3*1 *3*2 *3*3 *6*6 *1*6	35/79 (44.3) 26 (32.9) 7 (8.9) 5 (6.3) 5 (6.3) 1 (1.3)	45/192 (23.4) 83 (43.2) 31 (16.1) 24 (12.5) 4 (2.1) 3 (1.6) 1 (0.5) 1 (0.5)	0.39 (0.22-0.67) [C]
UGT1A4	*1*1 *1*2 *2*2 *1*3 *2*3	55/79 (69.6) 6 (7.6) 16 (20.3) 2 (2.5)	131/181 (72.4) 16 (8.8) 3 (1.7) 30 (16.5) 1 (0.6)	1.14 (0.64-2.04)
UGT1A6	*1*1 *1*2 *2*2 *1*3 *2*3	45/85 (52.9) 28 (32.9) 8 (9.4) 2 (2.4) 2 (2.4)	98/218 (45.0) 94 (43.1) 20 (9.2) 4 (1.8) 2 (0.9)	0.76 (0.46-1.26)
UGT1A10	*1*1	85/85 (100)	217/217 (100)	-
UGT2B4	*1*1 *1*2 *2*2	49/83 (59.0) 25 (30.1) 9 (10.9)	105/217 (48.4) 93 (42.9) 19 (8.7)	0.65 (0.39-1.09)
UGT2B7	*1*1 *1*2 *2*2	28/85 (32.9) 32 (37.6) 25 (29.5)	57/217 (26.2) 108 (49.8) 52 (24.0)	0.73 (0.42-1.25)
UGT2B15	*1*1 *1*2 *2*2	13/78 (16.6) 41 (52.6) 24 (30.8)	44/211 (20.9) 92 (43.6) 75 (35.5)	1.3 (0.67-2.61)
GSTA1	*A*A *A*B *B*B	37/85 (43.5) 38 (44.7) 10 (11.8)	88/211 (41.7) 89 (42.2) 34 (16.1)	0.93 (0.56-1.54)
GSTP1	Ile/ Ile Ile/Val Val/Val	31/85 (36.5) 39 (45.9) 15 (17.6)	90/209 (43.1) 86 (41.1) 33 (15.8)	1.32 (0.78-2.22)
GSTM1	+ null	38/85 (44.7) 47 (55.3)	100/215 (46.5) 115 (53.5)	1.08 (0.65-1.78)
GSTT1	+ null	60/85 (70.6) 25 (29.4)	165/214 (77.1) 49 (22.9)	1.40 (0.80-2.47)

[A] In both the FAP and control group, there is some missing data because of insufficient amount of DNA or unsuccessful PCR
[B] Genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with expected altered enzyme activities versus genotypes with expected normal enzyme activities).
[C] Statistically significant

Table 6: *Allele frequencies of UGT and GST polymorphisms in patients with FAP and controls.*

Alleles	Patients with FAP [A] (number/total)	Controls [A] (number/total)	OR (95% CI) [B]
*UGT1A1*1* *UGT1A1*28*	0.74 (126/170) 0.26	0.66 (286/432) 0.34	0.68 (0.46-1.02)
*UGT1A3*1* *UGT1A3*2* *UGT1A3*3* *UGT1A3*6*	0.64 (101/158) 0.28 0.08	0.52 (198/384) 0.38 0.09 0.01	0.60 (0.41-0.88) [C]
*UGT1A4*1* *UGT1A4*2* *UGT1A4*3*	0.84 (132/158) 0.05 0.11	0.85 (308/362) 0.06 0.09	1.12 (0.67-1.87)
*UGT1A6*1* *UGT1A6*2* *UGT1A6*3*	0.71 (120/170) 0.27 0.02	0.67 (294/436) 0.31 0.02	0.86 (0.59-1.27)
*UGT1A10*1* *UGT1A10*3* *UGT1A10*4*	1.0 (170/170)	1.0 (434/434)	-
*UGT2B4*1* *UGT2B4*2*	0.74 (123/166) 0.26	0.70 (303/434) 0.30	0.81 (0.54-1.21)
*UGT2B7*1* *UGT2B7*2*	0.52 (88/170) 0.48	0.51 (222/434) 0.49	0.98 (0.68-1.39)
*UGT2B15*1* *UGT2B15*2*	0.57 (89/156) 0.43	0.57 (242/422) 0.43	0.99 (0.68-1.43)
*GSTA1*A* *GSTA1*B*	0.66 (112/170) 0.34	0.63 (265/422) 0.37	0.87 (0.60-1.27)
*GSTP1*Ile* *GSTP1*Val*	0.59 (101/170) 0.41	0.64 (266/418) 0.36	1.20 (0.83-1.72)
GSTM1+ *GSTM1null*	0.45 (76/170) 0.55	0.47 (200/430) 0.53	1.08 (0.75-1.54)
GSTT1+ *GSTT1null*	0.71 (120/170) 0.29	0.77 (330/428) 0.23	1.40 (0.94-2.09)

[A] In both the FAP and control group, there is some missing data because of insufficient amount of DNA or unsuccessful PCR
[B] Genotypes were combined on the basis of an expected phenotype-genotype relationship (variant genotypes with expected altered enzyme activities versus genotypes withexpected normal enzyme activities).
[C] Statistically significant

Discussion

Without prophylactic colectomy, almost 100% of the patients with FAP will develop colorectal cancer by the age of forty [2]. In addition, these patients frequently develop duodenal adenomas and the prevalence of duodenal carcinomas is 2-5% [3, 7]. This process, although mainly driven by *APC* mutations, may be modulated to some extent by other factors, such as the enzymes involved in detoxification. Detoxification is pivotal in reducing the load of toxins and carcinogens entering the body via food, medication or lifestyle habits such as smoking and drinking alcohol. In addition to the germline *APC* mutations, an extra pressure of toxins or carcinogens may introduce additional somatic mutations, which can accelerate the processes of adenomatosis and carcinogenesis in patients with FAP. Several functional genetic polymorphisms with concomitant loss of enzyme activity have been described in the important detoxification enzymes of the GST and UGT families, which are mainly involved in the mucosal protection against carcinogens [11, 13]. We therefore investigated whether the polymorphisms in GST and UGT genes, which are associated with functional changes in the enzyme activity and may be involved in modulation of the phenotype, are differently distributed in patients with FAP versus age and gender matched controls.

A significantly different distribution was found for the genotypes of UGT1A3 in patients with FAP compared with controls. UGT1A3 is expressed in the liver, biliary and gastric tissues and in the intestine [18, 37, 38]. It catalyses the glucuronidation of important endogenous substances such as the steroid hormones estrone and 2-hydroxyestrone, the bile acid chenodexoycholic acid, as well as many exogenous compounds like primary amines, tertiary amines, hydroxylated benzo(a)yrenes, 2-acetylaminofluorenes, flavonoids, 7-hydroxycoumarins, opiods, anthraquinones and commonly prescribed drugs [37, 39, 40]. Iwai et al. [41] studied the different genotypes of UGT1A3 present in a Japanese population and measured the *in vitro* enzyme activity towards the substrate estrone, of the polymorphic variants of UGT1A3 expressed in COS cells. The UGT1A3*2 variant enzyme showed a significantly higher (269%) enzyme activity towards estrone compared to the most common UGT1A3*1

form, whereas the enzyme activity of the UGT1A3*3 variant was 21% higher compared to UGT1A3*1 (not significant). The variant allele UGT1A3*6 was not detected in either patients or controls under study here. Estrone is an endogenous female steroid hormone. Epidemiological and experimental evidence suggests an inverse association between ever use of oral contraceptives and the risk of colorectal cancer [42]. Interestingly, a recent case report showed complete adenoma regression in the colon of a patient with FAP, after the use of oral contraceptives [43]. Since UGTs including UGT1A3, are involved in the metabolism of female steroid hormones, the low UGT1A3 enzyme activity associated genotypes, more often present in patients with FAP, may possibly be a factor of importance in the modulation of adenomatosis. The current study revealed no difference between females and males in their UGT1A3 genotype distribution, although these subgroups are very small.

Polymorphisms in the UGT1A3 gene may also result in a decreased activity towards flavonoids. Flavonoids are dietary antioxidants widely found in fruits, vegetables, cereals, pulses, chocolate, and beverages [44]. The flavonoids such as quercetin, kaempferol and luteolin may play an important role in cancer prevention [45, 46]. Compared to the control group, patients with FAP more often have a genotype associated with a high conjugating enzyme activity towards the flavonoids. This may result in higher rates of inactivating and excretion of flavonoids in patients with FAP compared to controls, which could mean that the patients with FAP in general have less benefit from the dietary flavonoids. There are only two studies so far describing the effect of polymorphisms in the UGT1A3 gene [41, 46]. A better understanding of the effect of polymorphisms on different substrates could give new insights into the relationship between these polymorphisms and FAP.

In accordance with the results of Crabtree *et al.* [10] and Lamberti *et al.* [25], we found no differences between the genotype distribution of GSTT1 and GSTM1 in patients with FAP versus controls. In contrast, Spigelman *et al.* [22] found lower GSTM levels in the peripheral blood of patients with FAP.

The two genotype distributions tested here did not fit the Hardy-Weinberg criteria: the UGT1A4 (control group) and the UGT2B7 genotype (patient group). If genotype frequencies differ from the expected Hardy-Weinberg values, one or more of the assumptions of this model are being compromised. One assumption is that the population is large; the relatively small numbers of patients in this study may be an explanation for the above-cited disequilibrium in the two groups.

Although the difference in genotype distribution of UGT1A1 (patients with FAP vs. controls) lost significance after correction for multiple testing, it is tempting to speculate on the possible consequence of this observation. UGT1A1 is the only UGT involved in the breakdown of billirubin. The presence of one or two variant alleles of UGT1A1*28 was associated with lower bilirubin conjugating enzyme activity and higher serum levels of unconjugated billirubin [47], which is an established antioxidant. Higher levels of serum billirubin were associated with a lower risk of cardiovascular disease [48]. Furthermore, it has been suggested that higher serum levels of billirubin can reduce the risk of cancer [49]. Since the patients with FAP more frequently show the genotype with the normal UGT1A1 enzyme activity associated with lower serum billirubin levels, this could also contribute to an increased risk of (early) colorectal as well as duodenal tumours.

In conclusion, although the genotype distribution of *UGT1A3* is different in patients with FAP compared with controls, some critical remarks should be made. The number of patients included in this study is relatively small, only one out of the twelve polymorphisms investigated differs between patients with FAP and controls and there is no correlation with the polymorphisms and the Spigelman stage. Therefore the polymorphisms in UGTs and GSTs investigated here do not play an important modulating role in the inter-individual differences in the development of duodenal adenomas as noted in patients with FAP. Therefore, we conclude that the findings of this study can not be used in the clinical management of duodenal adenomas in patients with FAP.

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Chapter 4

Loss of extracellular E-cadherin in the normal mucosa of duodenum and colon of patients with familial adenomatous polyposis

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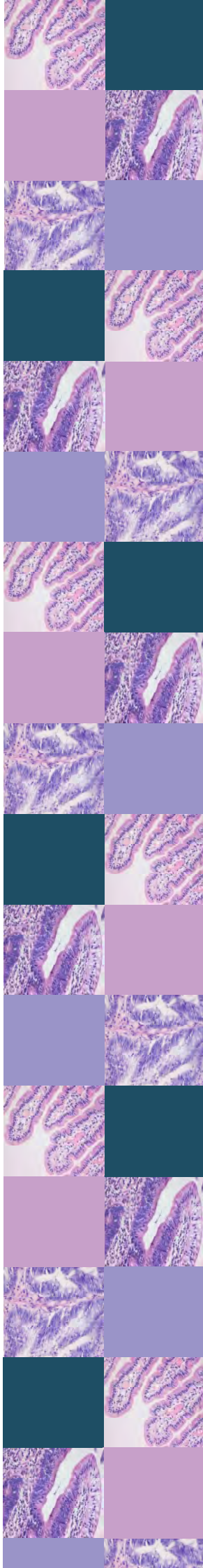


The duodenum is the main site for (pre-)malignant extra-colonic manifestations in patients with familial adenomatous polyposis. Changes in the E-cadherin / β-catenin complex play a pivotal role in the development of malignancies. Loss of E-cadherin has been described in association with loss of SMAD4. In order to elucidate the pathways leading to the development of duodenal adenomas in patients with FAP, the distributions of E-cadherin, SMAD4 and β-catenin were analysed. Furthermore, differences between the duodenum and colon were evaluated. Normal FAP duodenum (n=13) and FAP duodenal adenomas (n=50, total 21 patients) were compared with non-FAP duodenal adenomas (n=7) and normal non-FAP duodenum (n=15) by immunohistochemical staining for extracellular and intracellular E-cadherin, β-catenin and SMAD4. Colonic biopsies of 10 patients with FAP were also studied, as well as non-FAP colonic adenomas (n=26) and non-FAP normal colon (n=12). Compared to the intracellular component of E-cadherin that was present in all cases, a significant loss of extracellular E-cadherin was observed in both duodenal and colonic adenomas and in normal tissue of patients with FAP. Nuclear localisation of β-catenin was more often observed in duodenal FAP adenomas compared with non-FAP adenomas. Loss of nuclear SMAD4 was seen in the duodenum and to a larger extent in the colon of patients with FAP, as well as non-FAP patients.The loss of extracellular E-cadherin in the normal duodenal and colonic mucosa of patients with FAP might play a role in the high susceptibility of these tissues to (pre-)malignant transformation.

Introduction

Germline mutations in the tumour suppressor gene *Adenomatous polyposis coli* (*APC*) cause familial adenomatous polyposis (FAP) [1]. This autosomal dominant disorder is characterised by numerous colorectal adenomas, which will eventually progress to adenocarcinoma. Without prophylactic colectomy, FAP will inevitably lead to colorectal cancer at a relatively young age [2]. Additionally, patients with FAP run the risk of developing extra-colonic manifestations and the duodenum is the main site for these (pre-)malignant developments [2,3]. The prevalence of the mainly peri-ampullary located adenomas varies from 50% to over 90% [4-6]. The prevalence of duodenal carcinoma in patients with FAP is 2-5% [6-9] and, compared to the general population, the relative risk of duodenal adenocarcinoma is exceptionally high [3]. Together with desmoid tumours, duodenal carcinomas are now the leading causes of cancer-related mortality in FAP [10].

The mutations in the *APC* gene found in FAP lead to a disturbed function of the APC protein. The key tumour suppressor function of APC is to regulate the stability and cellular localisation of β-catenin [11]. β-catenin is a bifunctional protein; it has a crucial role in cell-cell adhesion [12] and a signalling role in the Wnt pathway [13]. Loss of functional APC in tumourigenesis deregulates the signalling properties of β-catenin leading to cytosolic accumulation and nuclear translocation. In the nucleus, it activates transcriptional targets such as c-MYC [14] and cyclin D1 [15]. In cell adhesion, β-catenin is crucial to the normal functioning of E-cadherin [16]. E-cadherin, the main adhesion molecule of epithelial cells, is a calcium-dependent transmembrane glycoprotein which mediates cell-cell adhesion [17]. Intracellular β-catenin binds to the cytoplasmic tail of E-cadherin forming an adhesion complex with the actin cytoskeleton [18]. E-cadherin/catenin complexes are also involved in differentiation, polarity, migration, proliferation, and survival of epithelial cells, which are all fundamental processes for the maintenance of normal intestinal epithelial architecture and function [19]. Mutations in the *E-cadherin* gene are associated with diffuse-type gastric cancer [20] and lobular breast carcinomas [21], and down regulation of E-cadherin expression has been described in many types of cancer. Moreover, loss or absence of E-cadherin is associated with aggressive

histopathological characteristics such as tumour invasiveness and metastasis [22]. In primary colorectal tumours, loss of E-cadherin is strongly linked to the loss of SMAD4 [23]. The tumour suppressor function of SMAD4 lies in its capacity to mediate the effects of TGF-β signalling superfamily [24]. Furthermore, in small intestinal adenocarcinomas, inactivating mutations of the *SMAD4* gene have been observed [25]. Recently, loss of E-cadherin was shown in colonic adenomas of patients with FAP, which suggests that it is an early event in the colonic adenoma-carcinoma sequence in FAP [26].

The aim of the current study is to evaluate the distribution of E-cadherin, β-catenin and SMAD4 in adenomas and normal duodenum of patients with FAP and to compare it with the patterns in non-FAP duodenal adenomas and normal non-FAP duodenum. In addition, the above-mentioned parameters are compared in the duodenum and colon of patients with FAP.

Materials & Methods

Patients and controls

In the period 1996-2004 duodenal specimens of patients with FAP were retrieved from the files of the Department of Pathology (Radboud University Nijmegen Medical Centre, the Netherlands). A total of 63 specimens including normal duodenal mucosa (n=13) and adenomas (n=50) of 21 patients were included. In addition, normal non-FAP duodenum (n=15) and non-FAP adenomas (n=7) from the same period were selected. No ampullary adenomas were included. To exclude HNPCC in the non-FAP patients with duodenal adenomas, immunohistochemical staining for the DNA mismatch repair proteins: MLH-1 MSH-2 and MSH-6 (see immunohistochemistry) was performed, for which all stainings were positive. Colonic specimens, both normal mucosa (n=3) and adenomas (n=22) of 10 of the patients with FAP, as well as non-FAP colonic adenomas (n=26) and normal non-FAP colon (n=12) specimens were included in the study. In 41% of the patients with FAP, the *APC* mutation was not known. Mutations in exons 12, 13, 14 and 15 were respectively observed in 5%, 27%, 5% and 17% of the patients with FAP. One

patient (5%) showed a *MYH* mutation. This study was approved by the local medical ethical review committee.

Histological evaluation

Adenomas were graded and classified according to histological type and degree of dysplasia by the method of Morson [27]. The slides were reviewed by two pathologists (JHJMvK & IDN).

Spigelman stage

All duodenal specimens of patients with FAP were graded according to Spigelman's stage, which is a semi-quantitive scoring system, expressing the severity of duodenal polyposis according to number, size, histology and degree of dysplasia of duodenal lesions (Table 1) [28].

Table 1: *Spigelman stage represents the severity in duodenal polyposis.*

Category	Points		
	1	2	3
Number of adenomas	1-4	5-20	> 20
Size of adenomas (mm)	1-4	5-10	> 20
Histology	Tubular	Tubulovillous	Villous
Degree of dysplasia	Mild	Moderate	Severe

Within each category, 1 to 3 points can be scored, and the sum of the score in the 4 categories results in the Spigelman stage. Stage 0, 0 points; Stage I, 1-4 points; Stage II, 5-6 points; Stage III, 7-8 points; Stage IV, 9-12 points.

Immunohistochemistry

Table 2 summarises the antibodies used in this study. In short, the paraffin–embedded tissues were cut into 4 μm sections. These sections were deparaffinised in xylene, rehydrated and incubated with 3% hydrogen peroxide/ PBS to block endogenous peroxidase. Antigen retrieval was performed by microwave exposure for 10 min in 10 mM citrate buffer (pH 6.0). The slides were allowed to cool down for at least 90 mins. After pre-incubation with 20% normal horse serum, sections were incubated overnight at 4° C with

the primary antibodies in PBS/ 1% Bovine Serum Albumin (BSA), followed by the incubations with a biotinylated secondary antibody and the avidin-biotin-peroxidase complex (Vector Laboratories Inc, Burlingame) respectively. Finally, 3,3'-diaminobenzidene (DAB) was used as the chromagen with haematoxylin counterstaining. Negative controls were constructed by omitting the primary antibody. For the DNA mismatch repair proteins the following antibodies (and antigen retrieval) were used:

- MLH1: 1:500, BD Biosciences, Europe, microwave exposure for 10 mins in 10 mM Tris/ 1 mM EDTA, pH 9.0;
- MSH2: 1:140, Merck Biosciences, Europe, microwave exposure for 10 mins in 10 mM Tris/ 1 mM EDTA, pH 9.0;
- MSH6: 1:1000, BD Biosciences, Europe, microwave exposure for 20 mins in 10mM citrate buffer, pH 6.0.

Table 2: *Primary antibodies used for immunohistochemistry.*

Antigen	Antibody	Duodenum dilution	Colon dilution	Animal source
E-cadherin extracellular	HECD-1, Takara Bio Inc., Japan	1:400	1:200	mouse
E-cadherin intracellular	Clone 36, BD Biosciences, Europe	1:1500	1:2000	mouse
B-catenin	Clone 14, BD Transduction Laboratories, Europe	1:8000	1:8000	mouse
SMAD4	B-8, Santa Cruz Biotechnology, USA	1:500	1:500	mouse

Immunofluorescent double staining

Immunofluorescent double staining was used to confirm light microscope findings and illustrative representation. Immunofluorescent double staining was performed, using antibodies against the extracellular and intracellular epitopes of E-cadherin. In short, after deparaffinising and rehydrating, the sections were washed overnight in PBS. Antigen retrieval was performed by microwave exposure for 10 mins in 10 mM citrate buffer (pH 6.0). The slides were allowed to cool down for at least 90 mins. Incubation with normal goat

serum (20%) in PBS for 10 mins blocked background staining. The sections were incubated with the first primary antibody (mouse anti-human HECD-1, Takara Bio Inc, Japan) at a dilution of 1:50 in PBS/ 1% BSA at 4˚ C overnight. The slides were incubated with a goat anti-mouse IgG antibody conjugated with alexa 594 (1:200, Molecular Probes, Inc, Eugene, USA) for 30 mins at room temperature. From this moment the sections were handled in the dark. After a second incubation with normal goat serum, the slides were incubated with the second primary antibody (mouse anti-human E-cadherin Clone 36, BD Biosciences, Europe) at a dilution of 1:1500 in PBS/ 1% BSA at 4º C overnight. The sections were then incubated with a goat anti-mouse IgG2a antibody conjugated with alexa 488 (1:200, Molecular Probes) for 30 mins at room temperature. The nuclei were stained with 4'-6-diamino-2-phenylindole (DAPI) for 20 secs. The slides were enclosed in Fluorteck (Euro-Diagnostica, Arnhem, the Netherlands) and examined with a fluorescence microscope (Leica, Solms, Germany).

Scoring method

Two categories for loss of membranous staining of E-cadherin were used: 1 = normal expression with maximal 5% loss and 2 = 50-90% loss of E-cadherin, a self-designed scoring method. This scoring method was derived from the first scoring into four categories: 1, no loss; 2, low loss; 3, moderate loss; and 4, high loss. After this scoring, it was evident that all samples could be classified into the two categories: no to low loss (1 & 2) and moderate to high loss (3 & 4). These categories represent the percentages of loss observed, namely: 1= normal with maximal 5% loss and 2 = 50 – 90% loss, as described above. Nuclear loss of SMAD4 could also be put into the categories 1 and 2 and focal loss of nuclear SMAD4 staining was noted. Beta-catenin was scored for membranous, cytoplasmic or nuclear expression, should any part of the specimen show this expression. Localisation was considered cytoplasmic if membranous staining was totally absent. The slides were independently reviewed by two of the authors (MB & MJEMG). In less than 5% of the slides differences between the two observers occurred, these specimens were re-evaluated by the two observers together and consensus was obtained.

Statistical analysis

Fisher's exact test was used to compare the loss of E-cadherin and SMAD4 between the different groups. One-way ANOVA was performed to examine the relation between the age of the subjects and other categorical parameters. Associations between the categorical parameters were tested with the Chi-square test. For statistical correctness, analyses were also performed with one randomly chosen sample for each patient. These analyses gave the same results as with multiple sampling of one patient. $P < 0.05$ was considered to be statistically significant (SSPS 12.0.1 for Windows 2003, SPSS Inc., Chicago, USA)

Results

Patient characteristics

Specimens were collected at several time points from almost all patients with FAP. Table 3 lists the duodenal and colonic biopsies of the patients with FAP included in this study and summarises data on age, histological type and degree of dysplasia. The average age of the non-FAP patients is 62 ± 7 years. Patients with FAP and duodenal adenomas were significantly younger compared with the non-FAP patients (average age 50 ± 13 vs. 62 ± 7 years, $p = 0.03$). The age of patients with FAP with a normal duodenum did not differ from that of the non-FAP patients with a normal duodenum ($p = 0.174$).

At an average age of 28 ± 11 years at the moment of surgery, all patients with FAP underwent colectomy; 52.4% with an ileal-rectal anastomosis, 33.3% with an ileal pouch-anal anastomosis and 14.3% with an ileal stoma. The average interval between colectomy and the sampling of duodenal specimens used for this study was 15.9 ± 7 years.

The Spigelman stages classify the severity of duodenal polyposis into five categories (0- IV). Samples in this study were classified as stage 0: 20.6%, stage I: 6.3%, stage II: 36.5%, stage III: 28.6% and stage IV: 8%. The Spigelman's stage increased with the increasing age of the patients with FAP (Spigelman I: 39 ± 5 years versus Spigelman III: 56 ± 12 years, $p = 0.04$).

Characteristics of the colonic biopsies of the patients with FAP are also listed in Table 3. The average age of the non-FAP patients with normal colon or colonic adenomas is 54 ± 19 years and 67 ± 11 years respectively.

Table 3: *Characteristics of patients with FAP for both duodenal and colonic biopsies.*

ID	**Normal duodenum**	**Duodenal adenomas**			**Normal colon**	**Colonic adenomas**		
	Age	**Age**	**Type**	**Dysplasia**	**Age**	**Age**	**Type**	**Dysplasia**
F1					21	21	TA	II
F2	57 (2x)	44, 51, 52 (2x), 53 (2x) 54 (3x), 55, 58	TA (2x) TV (5x) VA (4x)	I (2x) II (9x)		34, 42, 49	TA (3x)	I (2x), II
F3		35, 39	TA, TV	I (2x)		29	TA	I
F4		31	TA	I		48 (2x), 56	TA (3x)	I, II (2x)
F5		26, 28	TA (2x)	I (2x)		17 (3x), 23	TA (4x)	I (4x)
F6		40	TA	II		28 (2x)	TA (2x)	I (2x)
F7		41, 43 (2x)	TA (3x)	I (3x)				
F8	55, 60					14, 15 (2x), 17 (2x)	TA (5x)	I (2x), II (2x), III
F9	56				34 (2x)			
F10	45	48	TA	I		26 (2x)	TA, TV	I, II
F11		73	TA	I		36	TA	I
F12		31	TA	I				
F13	43, 44, 45	41 (3x), 43 (3x), 44	TA (7x)	I (7x)				
F14		67 (3x), 68 (3x), 69	TA TV (6x)	I II (6x)				
F15		38	TA	II				
F16	29, 32							
F17		48, 50	TA, TV	I, II				
F18		65 (2x)	TA (2x)	I (2x)				
F19	24							
F20		59, 60, 61, 62, 63	TA TV (2x) VA (2x)	II (5x)				
F21		36, 37	TA, TV	I, II				
F22	30	31	TV	I				

Type: TA = Tubular adenoma, TV = Tubulovillous adenoma, VA = Villous adenoma
Dysplasia: I = mild, II = moderate, III = severe

Histological evaluation of patients with FAP

In 21% of the duodenal biopsies from patients with FAP, the duodenum showed no abnormalities. Tubular adenomas were observed in 42% of the biopsies, 27% showed tubulovillous adenomas and 10% villous adenomas. The age of the patients with tubular adenoma was significantly lower compared with patients with tubulovillous and villous adenomas (45 ± 13 vs.

55 ± 13 and 55 ± 5 years respectively; p = 0.04). Tubular adenomas were observed earlier after colectomy (17 ± 10 years) compared with tubulovillous adenomas (29 ± 14 years, p = 0.01) and villous adenomas (22 ± 7 years). Moderately graded dysplasia was present in only 15% of all tubular adenomas compared to 100% of all villous adenomas (p < 0.0001). Moderately graded dysplasia was more often observed in older patients compared with mildly graded dysplasia (56 ± 9 vs. 44 ± 13 years, respectively; p = 0.001). In addition, mildly graded dysplasia was observed earlier after colectomy compared with moderately graded dysplasia (16 ± 10 vs. 28 ± 11 years, p = 0.001).

The colonic adenomas were tubular in 95% and tubulovillous in 5% of the cases. Mild dysplasia was seen in 64% of the cases, moderate dysplasia in 32% and severe dysplasia in 4%.

Histological evaluation of non-FAP patients

The non-FAP patients with duodenal adenomas showed tubular adenomas with mild graded dysplasia in 71% of the cases. Due to the small number of patients (n=7), statistical analyses were not performed.

Colonic adenomas were tubular in 73% and tubulovillous in 27% of the cases. Mild dysplasia was seen in 67% of the cases, moderate dysplasia in 27% and severe dysplasia in 6%.

Loss of E-cadherin

All specimens used in this study showed membranous staining for E-cadherin. For the extracellular component of E-cadherin only two categories were observed: normal staining with a maximum of 5% loss and specimens showing 50–90% loss. Compared to the intracellular component of E-cadherin that was present in all cases a significant loss of extracellular E-cadherin was observed in FAP duodenal adenomas (0% and 54% respectively, p < 0.001). In 43% of the non-FAP adenomas, loss of extracellular E-cadherin was observed compared with 0% loss of the intracellular component. Loss of extracellular E-

cadherin was also seen in the morphologically normal duodenum of patients with FAP (54% versus 7% in normal non-FAP duodenum, p = 0.01).

Patients with FAP and adenomas showing normal staining of E-cadherin were older at the moment of colectomy (31 ± 9 years) compared with patients with 50 – 90% loss (26 ± 9 years, p = 0.03). Loss of E-cadherin was not associated with the age of the patients, Spigelman stage, *APC* mutation, type of adenoma or degree of dysplasia. Furthermore, no intra-individual changes in loss of extracellular E-cadherin could be observed in the multiple samples of the patients. Immunofluorescent double staining of the extracellular and intracellular epitopes of E-cadherin in normal FAP duodenum and normal non-FAP duodenum is depicted in Figure 1. Figure 2 shows light microscopic images of the extracellular and intracellular epitopes of E-cadherin in FAP and non-FAP duodenum.

Similar to the duodenum, loss of extracellular E-cadherin was observed in FAP colonic adenomas; however, this loss was not seen in the non-FAP colonic adenomas (64% and 4% respectively; p < 0.001). In addition, the normal colon of patients with FAP showed loss of extracellular E-cadherin (100% versus 0% in normal non-FAP colon, p < 0.001). The intracellular E-cadherin was present in the colon in all cases, as it was in the duodenum. Table 4 summarises the results of the immunohistochemical findings in the duodenum compared to these findings in the colon.

Beta-catenin localisation

Membranous localisation of β-catenin was seen in 96% of the FAP duodenal adenomas, 86% of the non-FAP duodenal adenomas and 100% of normal FAP and non-FAP duodenum. Cytoplasmic localisation was not present in normal non-FAP duodenum. Cytoplasmic localisation was observed in 8% of normal FAP mucosa, 32% of FAP adenomas and 14% of non-FAP adenomas. Nuclear localisation was more often observed in the FAP adenomas compared to the non-FAP duodenal adenomas (84% and 14%, respectively; p = 0.001). There were no differences in nuclear localisation between normal FAP and non-FAP duodenum. Membranous, cytoplasmic and nuclear localisation of β-

catenin is depicted in Figures 3A & B. Distribution of β-catenin was not associated with degree of dysplasia. However, these observations of β-catenin in relation to dysplasia are based on the general estimation of dysplasia in the whole biopsy. If observed in more detail, in a slide-by-slide comparison, the localisation of β-catenin seems to be directly related to the type of dysplasia. In focal areas with mild dysplasia, β-catenin is located membranous, compared to cytoplasmic and nuclear localisation in areas with moderate to severe dysplasia ($p = 0.004$).

Membranous localisation of β-catenin was visible in all normal FAP and non-FAP colon. Nuclear localisation was observed in 73% and 85% of the FAP adenomas and non-FAP adenomas respectively. Cytoplasmic localisation was observed in 32% and 27% of the FAP and non-FAP adenomas respectively. The distribution of β-catenin was associated with the degree of dysplasia: adenomas with a high degree of dysplasia more frequently showed cytoplasmic localisation of β-catenin (mild dysplasia vs. severe dysplasia, 21% and 50% respectively; $p = 0.02$).

Loss of SMAD4

In the FAP duodenal adenomas, 42% of the cases showed loss and the loss was seen focally in 21% of the cases. There was no difference between FAP adenomas and non-FAP adenomas. In normal FAP and non-FAP duodenum, 92% respectively 93% of the cases showed no loss of SMAD4 and there was no focal reduction of nuclear SMAD4. In the FAP adenomas, loss of nuclear SMAD4 was associated with loss of extracellular E-cadherin ($p = 0.05$). Loss of SMAD4 was correlated with a high Spigelman's stage ($p = 0.015$). Normal staining, focal appearance and loss of SMAD4 is depicted in Figures 3C-F.

Loss of nuclear SMAD4 was observed in both FAP and non-FAP colonic adenomas (82% and 85% respectively). In the normal colon 100% (FAP) and 92% (non-FAP) of the samples showed loss of nuclear SMAD4.

Figure 1: *Immunofluorescent double staining of E-cadherin in normal FAP duodenum and normal non-FAP duodenum, magnification 400x. A: intracellular epitope of E-cadherin in normal FAP duodenum, B: extracellular epitope of E-cadherin in normal FAP duodenum, C: merge colour of both intracellular and extracellular epitopes of E-cadherin in normal FAP duodenum, D: intracellular epitope of E-cadherin in normal non-FAP duodenum, E: extracellular epitope of E-cadherin in normal non-FAP duodenum, F: merge coulour of both intracellular and extracellular epitopes of E-cadherin in normal non-FAP duodenum.*

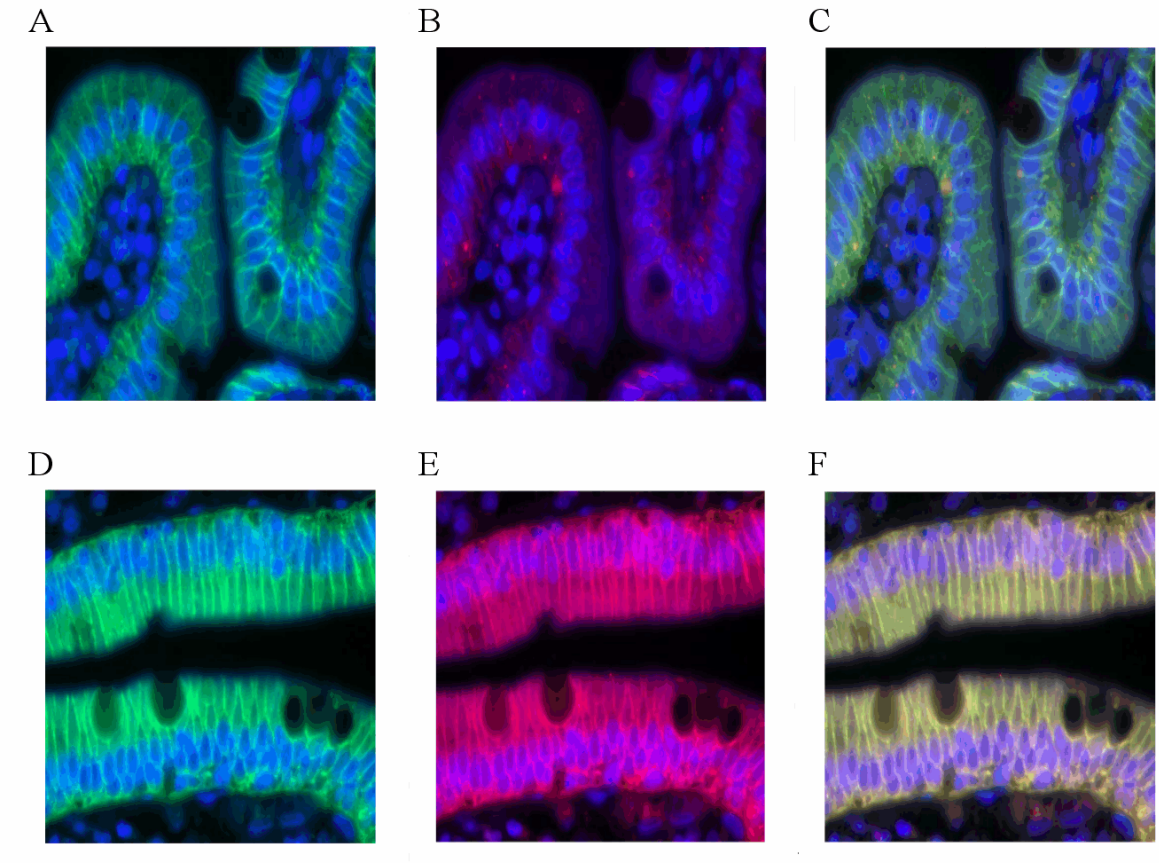


Figure 2: *Immunohistochemical staining for the extracellular and intracellular epitopes of E-cadherin in FAP and non-FAP duodenum, magnification 400x. A: extracellular E-cadherin loss in normal FAP duodenum, B: normal staining of extracellular E-cadherin in normal non-FAP duodenum , C: extracellular E-cadherin loss in adenoma of FAP duodenum, D: extracellular E-cadherin loss in adenoma of non-FAP duodenum, E: normal staining of intracellular E-cadherin in normal FAP duodenum, F: normal staining of intracellular E-cadherin in normal non-FAP duodenum, G: normal staining of intracellular E-cadherin in adenoma of FAP duodenum, H: normal staining of intracellular E-cadherin in adenoma of non-FAP duodenum. Staining results shown here, are also representative for the FAP and non-FAP colon.*

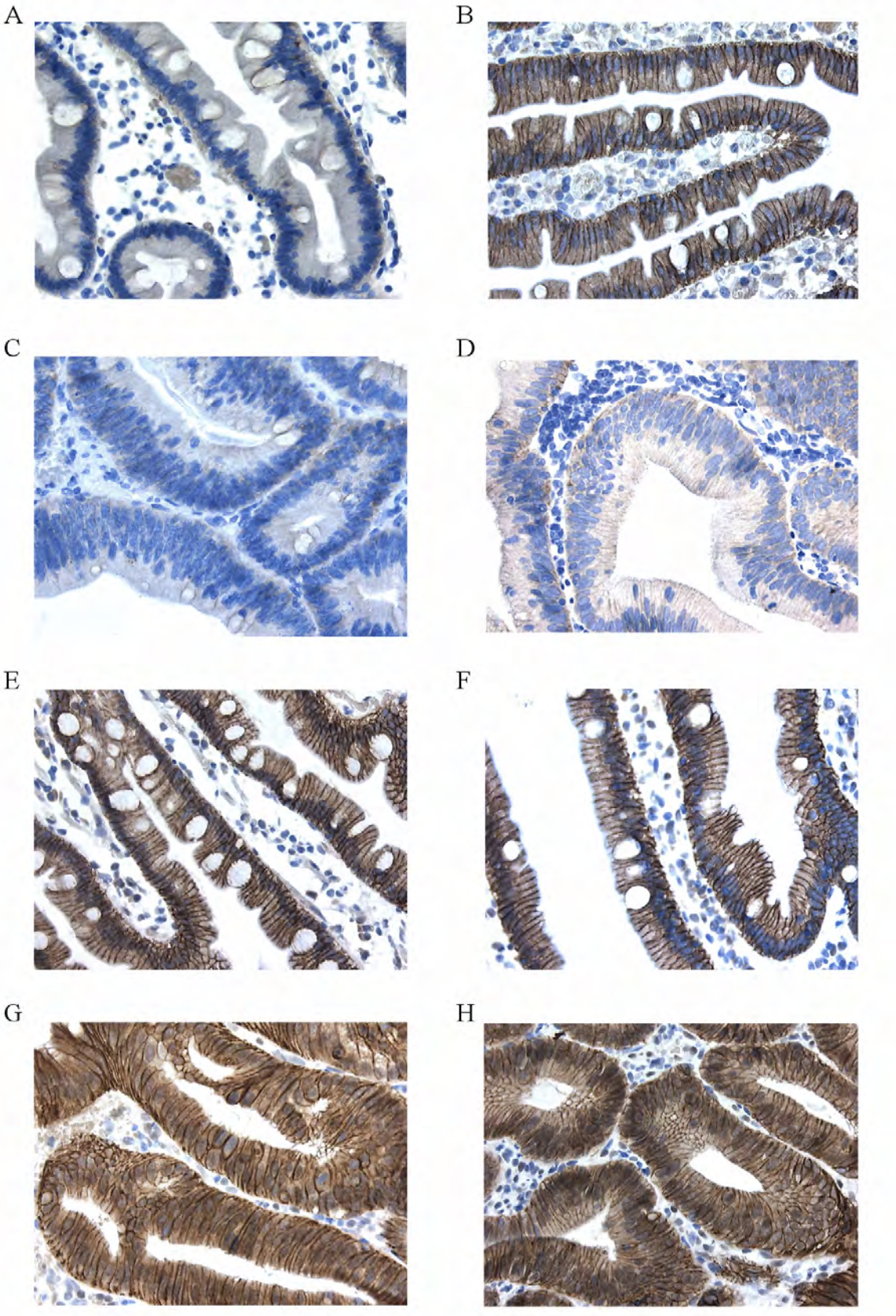
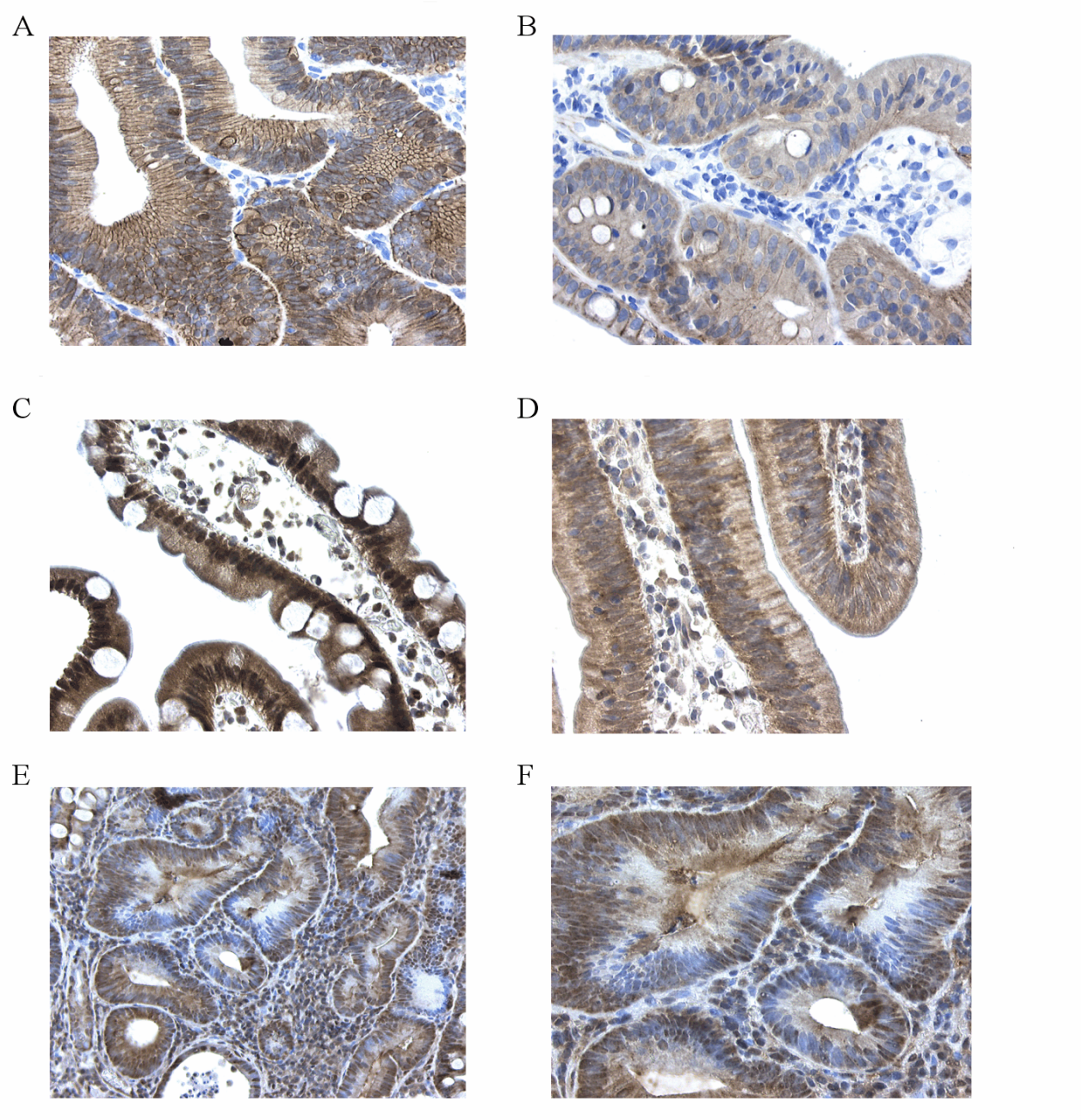

A
B
C
D
E
F
G
H

Figure 3 *Immunohistochemical staining for β-catenin and SMAD4 in FAP duodenum, magnification 400x. A: membranous and nuclear localisation of β-catenin in adenoma, B: cytoplasmic localisation of β-catenin in adenoma, C: nuclear staining of SMAD4 in normal mucosa, D: loss of nuclear SMAD4 in adenoma, E: focal appearance of SMAD4 in adenoma 200x, F: focal appearance of SMAD4 in adenoma . Staining results shown here, are also representative for non-FAP duodenum and FAP and non-FAP colon.*



Duodenum versus colon

In patients with FAP, loss of extracellular E-cadherin was observed in morphologically normal mucosa of the duodenum (54%) and colon (100%), as well as in the adenomas (duodenum: 52%, colon: 64%). In the non-FAP patients, there was extracellular loss of E-cadherin in the duodenal adenomas (43%) but not in the colonic adenomas (4%). Almost no loss of extracellular E-cadherin was found in the morphologically normal mucosa of non-FAP patients. Intracellular E-cadherin was found to be present in all cases in the duodenum and colon (Table 4).

In normal FAP colon, cytoplasmic localisation of β-catenin was observed far more often (100%) compared to normal FAP duodenum (8%). Nuclear localisation of β-catenin was often present in both normal non-FAP and FAP duodenum (47% and 54%), whereas it was absent in normal non-FAP colon (0%) and only present in one of the FAP colons. In non-FAP adenomas, nuclear localisation is more often seen in the colon (85%) compared to the duodenum (14%) (Table 4).

With the exception of non-FAP adenomas, the loss of nuclear SMAD4 was observed significantly more often in the colon than in the duodenum (Table 4).

Table 4: *Results of immunohistochemical staining for extracellular and intracellular E-cadherin, β-catenin and SMAD4 in normal mucosa of patients with FAP, normal non-FAP, FAP adenomas and non-FAP adenomas in the duodenum and colon.*

		E-cadherin loss		β-catenin presence			SMAD4 loss
		Intracellular loss (%)	Extracellular loss (%)	Membranous (%)	Cytoplasmic (%)	Nuclear (%)	Nuclear loss
FAP normal	Duodenum (n=13)	0	54	100	8	54	8
	Colon (n=3)	0	100	100	100	33	100
P value		*ns*	*ns*	*ns*	*0.009*	*ns*	*0.007*
Non-FAP normal	Duodenum (n=15)	0	7	100	0	47	7
	Colon (n=12)	0	0	100	0	0	92
P value		*ns*	*ns*	*ns*	*ns*	*0.01*	*0.001*
FAP adenomas	Duodenum (n=50)	0	52	96	32	84	42
	Colon (n=22)	0	64	100	32	73	82
P value		*ns*	*ns*	*ns*	*ns*	*ns*	*0.004*
Non-FAP adenomas	Duodenum (n=7)	0	43	86	14	14	57
	Colon (n=26)	0	4	100	27	85	85
P value		*ns*	*0.006*	*ns*	*ns*	*0.005*	*ns*

Ns: not significant

Discussion

In this study, loss of extracellular E-cadherin has already been observed in the normal duodenum of patients with FAP, whereas this loss in non-FAP patients is first observed in the adenoma stage, suggesting that loss of extracellular E-cadherin is an early event in the (pre-)malignant transformation of the FAP duodenum. These findings are in accordance with the results of Biasco *et al.*, who observed changes in the adherens junctions in normal duodenal mucosa of patients with FAP [29]. Other cellular abnormalities were also observed in the normal duodenum of patients with FAP, such as a higher proliferation index [29-30], increased numbers of Paneth cells, endocrine cells in the crypts of normal FAP duodenum [31] and increased levels of cyclooxygenase-2 [32]. Although loss of extracellular E-cadherin was observed in the normal mucosa of both duodenum and colon of patients with FAP, the morphology showed no abnormalities. The morphological integrity is possibly maintained by other adhesion molecules, such as Ep-CAM, which showed no loss in this study (data not shown).

The prevalence of non-FAP duodenal adenomas is low [33]; the number of patients included in this study is therefore small. Similar to the duodenal adenomas of patients with FAP, loss of extracellular E-cadherin in non-FAP adenomas was found. Although presumably the duodenal mucosa out of which the adenomas develop from the beginning differs with respect to proliferation and other cell kinetics [29-31], the mechanism of loss of extracellular E-cadherin in adenomas seems similar.

In contrast to the duodenal adenomas, almost no loss of extracellular E-cadherin was observed in non-FAP colonic adenomas. However, differences in carcinogenesis pathways in the small intestine and colon have already been described. *APC* mutations, which play a pivotal role in the initiation phase of colorectal tumourigenesis, are hardly present in small intestinal adenocarcinoma [34-36], suggesting a possible different genetic pathway. Because the loss of extracellular E-cadherin is seen in the duodenal adenomas of patients with FAP as well as non-FAP patients, the direct role of the *APC* mutations in these observations is also questionable.

Indirect evidence for the non-involvement of *APC* mutations is derived from the fact that mutations in the *APC* gene, as are present in FAP, could shift the cellular equilibrium of β-catenin away from the adherens junctions towards the pool of free β-catenin. However, we found no evidence of disturbance in the expression of the intracellular part of E-cadherin to which β-catenin is bound. Apparently, the deregulation of β-catenin by *APC* mutations does not necessarily lead to a disturbance in the cell adhesion complex. In addition, *in vitro* experiments demonstrated that E-cadherin-mediated cell adhesion is maintained after elimination of β-catenin. This suggests a regulatory role for β-catenin instead of a mechanical role in cell adhesion [37].

In patients with FAP, differences between the duodenum and the colon are evident: the prevalence of duodenal adenomas and carcinomas is 50-90% and 2-5% [2, 6-9], compared to corresponding values of 100% and 90% respectively in the colon [38]. Moreover, in contrast to the colon, the correlation between the severity of duodenal polyposis and mutations in the *APC* gene is less well understood [39]. These differences point to the possibility of a different mechanism underlying the development of adenoma and carcinoma in the small intestine compared to the large intestine. The difference between the duodenum and the colon was also outlined in the loss of SMAD4. Loss of nuclear SMAD4 was seen in almost every colonic sample in contrast to the duodenum. These observations are very difficult to explain, although it should be taken into consideration that the number of normal non-FAP colonic samples is very small.

Reduced or absent staining of the extracellular part of E-cadherin, is also seen in diffuse gastric cancer [20] and lobular breast cancer [21], diseases both associated with mutations in the extracellular domain of the *E-cadherin* gene. One single amino acid substitution in the extracellular domain of E-cadherin has been shown to result in functional consequences for cell adhesion [40]. However, in colorectal carcinomas a reduced staining of extracellular E-cadherin was also observed which was not related to any mutation in the *E-*

cadherin gene [41]. Other possible explanations for the loss of E-cadherin are: steric hindrance by episialin [42] or proteoglycans [43] or the involvement of matrix metalloproteinase-7 (MMP-7). *In vitro*, this enzyme is capable of the ectodomain cleavage of E-cadherin [44, 45].

MMP-7 mRNA has been detected in adenomas and carcinomas of the colon [46, 47]. In FAP, the expression of MMP-7 in normal colon mucosa was 25 times higher and in colon adenomas as much as 700 times higher, compared to the levels in normal colon mucosa of controls [48]. Furthermore, MMP-7 is a target gene for β-catenin [49]. Since MMP–7 is often increased in the development of tumours, the non-FAP adenomas could also show loss of extracellular E-cadherin. However, since MMP-7 may be over expressed in normal tissue of patients with FAP, the loss of extracellular E-cadherin can also occur in the normal mucosa of patients with FAP. In addition, *APC* mutations were linked to an increased secretion of MMP-7 from Paneth cells [50], which are present in higher numbers in the normal duodenum of patients with FAP [31]. We did not include MMP-7 immunohistochemistry results in this study, as staining with a commercial antibody (clone 6A4, R&D systems, USA) produced inconsistent results.

In this study, no loss of the intracellular epitope of E-cadherin was observed using the clone 36 antibody. This antibody has been described to show cross-reactivity with P-cadherin [51]. However, E-cadherin is the main cadherin protein expressed on the cell membranes of healthy epithelial cells, whereas P-cadherin is not expressed in a normal colon [52]. We found no loss of E-cadherin staining in the normal colon using the clone 36 antibody for E-cadherin and since P-cadherin is not present in normal colon [52], we still conclude that E-cadherin is present.

Loss of extracellular E-cadherin is associated with the development of diffuse gastric cancer [20] and lobular breast cancer [21], which suggests a direct role of E-cadherin loss in the development of malignancies. Furthermore, in this study loss of duodenal extracellular E-cadherin is associated with a younger age of

the patients with FAP at the moment of colectomy, suggesting a more severe course of the disease.

In conclusion, a significant loss of extracellular E-cadherin was demonstrated in normal mucosa and corresponding adenomas of the duodenum and colon of patients with FAP. This loss may play a role in the high susceptibility of both duodenum and colon for (pre-)malignant transformation in these patients.

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Chapter 5

Chromosomal and methylation alterations in sporadic and familial adenomatous polyposis-related duodenal carcinomas.

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Primary carcinomas of the small intestine are rare and the mechanism of their pathogenesis is poorly understood. Patients with familial adenomatous polyposis (FAP) have a high risk of developing duodenal carcinomas. The aim of this study is to gain more insight into the development of duodenal carcinomas. Therefore, five FAP-related duodenal carcinomas were characterized for chromosomal and methylation alterations, which were compared to those observed in sporadic duodenal carcinomas. Comparative genomic hybridization (CGH) and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was performed in ten primary sporadic and five primary FAP-related duodenal carcinomas. In the FAP-related carcinomas, frequent gains were observed on chromosomes 8, 17 and 19, whereas in sporadic carcinomas they occurred on chromosomes 8, 12, 13 and 20. In 60% of the sporadic carcinomas, gains in the regions of chromosome 12 were observed which were absent in the FAP-related carcinomas (p = 0.04). Hypermethylation was observed in the *immunoglobulin superfamily genes member 4* (*IGSF4)*, *TIMP metallopeptidase inhibitor 3* (*TIMP3)*, *Estrogen receptor 1* (*ESR1)*, *adenomatous polyposis coli* (*APC)*, *H-cadherin (CDH13)* and *paired box gene 6* (*PAX6)* genes. Hypermethylation of *PAX6* was only observed in FAP-related carcinomas (3/5) and not in sporadic carcinomas (p=0.02). In conclusion, in contrast to sporadic duodenal carcinomas, gains on chromosome 12 were not observed in duodenal carcinomas of patients with FAP. Identification of the genes in these regions of chromosome 12 could lead to a better understanding of the carcinogenesis pathways leading to sporadic and FAP-related duodenal carcinomas. Furthermore, hypermethylation seems to be a general feature of both FAP-related duodenal carcinomas as well as sporadic duodenal carcinomas with the exception of the *PAX6* gene, which is methylated only in FAP-related carcinomas.

Introduction

Although the small intestine is located between the stomach and the colon, both regions with a high cancer risk, carcinomas of the small bowel are surprisingly rare. In the Netherlands, the average annual incidence rate of small bowel carcinomas is approximately 1 case per 100,000 inhabitants, compared to 49 for colorectal cancer and 14 for gastric cancer [1]. In contrast to colorectal cancer, the molecular pathogenesis of small bowel tumours is rarely the subject of research. In the small bowel, the most frequent site for the development of adenocarcinomas is the duodenum (± 50%), followed by the jejunum (± 25%) and the ileum (± 13%) (rest is not specified) [2-4]. Patients with familial adenomatous polyposis (FAP) [5], Crohn's disease [6], coeliac disease [7] or hereditary nonpolyposis colorectal cancer (HNPCC) [8] are known to have a higher risk of developing small bowel carcinomas.

In patients with FAP, the duodenum is the main site for malignant transformation of mucosal cells in the small intestine. FAP is an autosomal dominant disorder caused by germline mutations in the tumour suppressor gene a*denomatous polyposis coli* (*APC*) [9]. Patients initially develop hundreds to thousands of colorectal adenomas. Without prophylactic colectomy, FAP will inevitably lead to colorectal cancer at a relatively young age [10]. Nowadays, with increased survival due to prophylactic colectomy, problems of extra-colonic manifestations in these patients become apparent [11]. At present, the prevalence of duodenal carcinoma in patients with FAP is 2-5% [11-14]. Compared to the general population, the relative risk of duodenal adenocarcinoma is exceptionally high (relative risk, 331; 95% confidence limits: 133 -681) [15]. Together with desmoid tumours, duodenal carcinomas are now the leading causes of cancer-related mortality in patients with FAP [16].

DNA changes are crucial steps in tumour initiation and progression [17]. Next to mutations in oncogenes and tumour suppressor genes, alterations in DNA copy numbers and DNA methylation patterns have been observed as common changes in colorectal and gastric cancer. Copy number changes can lead to increased or decreased gene expression whereas mutations can have an

activating or inactivating effect. Besides these genetic changes, epigenetic changes, such as DNA-methylation, may result in altered gene expression levels. Usually, aberrant methylation of normally unmethylated CpG-rich areas, also known as CpG-islands, which are located in the promoter regions of genes, have been associated with transcriptional inactivation of important tumour suppressor genes, DNA repair genes or metastasis inhibitor genes [18, 19].

Until now, only a few reports have been published on chromosomal changes or methylation of DNA with respect to the carcinogenesis of the small intestine [20-23], which lead us to use global screening methods in this study. Comparative genomic hybridization (CGH) enables detection of chromosomal gains and loses, and can be applied to fixed tissue samples. Major advantages of CGH are that is does not require pre-knowledge about the genetic constitution of the tumour tissue and the entire genome is analysed in one single experiment [24]. Multiplex ligation-dependent probe amplification (MLPA) has been accepted as a simple and reliable method for detection of copy number changes in paraffin-embedded tumour tissue [25]. Recently, this technique was adjusted allowing methylation specific analysis (MS-MLPA) [26]. A major advantage over the conventionally used techniques analysing the methylation status is that multiple loci can be analysed simultaneously using formalin-fixed and paraffin-embedded tissue.

So far, no comparison has been made between chromosomal and methylation alterations in sporadic versus FAP-related duodenal carcinomas. The aim of this study is to gain more insight into the development of duodenal carcinomas. FAP-related duodenal carcinomas were therefore characterised for chromosomal and methylation alterations, which were compared to those observed in sporadic duodenal carcinomas.

Materials & Methods

Patients

In the period 1991-2004, five primary duodenal carcinomas of patients with FAP (average age 54 ± 9 years) and ten primary sporadic duodenal carcinomas (average age 63 ± 19 years) were retrieved from the files of the Departments of

Pathology (Radboud University Nijmegen Medical Centre, Nijmegen and Rijnstate Hospital, Alysis, Arnhem, the Netherlands). Non-neoplastic duodenal tissue was included to serve as control DNA in the comparative genomic hybridization. The patients with FAP were diagnosed on their clinical characteristics: namely the presence of hundreds of adenomas in their colon. The local medical ethical review committee approved this study.

Histological evaluation of the carcinomas

Classification of the carcinomas was performed using the WHO guidelines; a tumour was considered mucinous when over 50% of the adenocarcinoma was mucinous [27]. Histological differentiation was categorised into well, moderately, poorly, or undifferentiated adenocarcinomas based on the part of poorest differentiation in the tumour, excluding the invasive front [28]. Growth pattern and peritumoural inflammation was assessed according to Jass *et al*. [29] Fibroblastic reaction and intratumoural inflammation were scored as none, little, moderate or extensive.

DNA isolation

At least 10 sections (20 μm) of macro-dissected (>80% tumour cells) formalin-fixed and paraffin-embedded tumour tissue were collected and incubated in 125 μl P-buffer (50 mM Tris/HCl pH 8.2, 100 mM NaCl, 1 mM EDTA, 0.5% (v/v) Tween-20, 0.5% (v/v) NP40, 20 mM DTT) for 15 min at 90 °C. Protein digestion was performed by adding proteinase K (Roche Diagnostics GBMH, Mannheim, Germany) with a final concentration of 0.5 mg/ml. The samples were incubated at 55° C for 24 hrs, followed by incubation at 37° C for 48 hrs with addition of 5 μl fresh proteinase K (20 mg/ml) every 24 hrs. Subsequently, DNA was purified using the DNeasy tissue kit (Qiagen, Venlo, the Netherlands). The isolation was performed according to the instructions of the manufacturer with the modifications of adding 250 μl ethanol in step 4 and repeating step 7 with buffer AW2. The DNA concentration was measured by using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, USA).

Comparative genomic hybridization: CGH

All carcinomas were genetically characterised by conventional comparative genomic hybridization (CGH) detecting copy number changes > 2 Mb as described previously [30-32]. In short, all DNA samples isolated from control- and tumour tissues were labeled by nick-translation with digoxigenin-dUTP and biotin-dUTP, respectively (Roche Molecular Biochemicals, Almere**,** the Netherlands), and precipitated in the presence of 50X human COT-1 DNA (Gibco BRL Life Technologies Inc., Gaitherburg, USA) and herring sperm DNA (Invitrogen, Carlsbad, USA). The probe and the metaphase slides were denatured simultaneously. After hybridisation and post-hybridisation washes, biotin and digoxigenin were detected using streptavidin-FITC and sheep-anti-digoxigenin-TRITC (Roche Molecular Biochemicals). The chromosomes were counterstained with 4,6'-diamino-2-phenylindole-dihydrochloride (DAPI) (Merck, Darmstadt, Germany) and the slides were mounted in Fluoroguard (Biorad, Veenendaal, The Netherlands). For CGH analysis, Quips CGH software (Applied Imaging, Newcastle upon Tyne, United Kingdom) was used. Detection thresholds for losses and gains were set at 0.8 and 1.2, respectively. For clear copy number changes, the thresholds were 0.6 and 1.4 and a ratio larger than 1.6 indicated high copy number amplifications. The average of approximately ten metaphases was used to calculate the ratio profiles of the chromosomes.

Methylation–specific multiplex ligation-dependent probe amplification: MS-MLPA

This technique uses multiple probe sets each consisting of 2 oligonucleotides, both containing a sequence-specific region used for hybridisation to the genomic test DNA, tagged with common tails complementary to an universal primer set. One of both oligonucleotides additionally contains a stuffer sequence of a characteristic length, allowing separation of the individual loci (probe sets) analysed. The probe mix, containing multiple probe sets is hybridised onto the genomic test DNA. In one part of the sample adjacently hybridised oligonucleotides are joint through ligation, whereas for the other half of the sample ligation is combined with a methylation sensitive restriction enzyme *HhaI* (recognition site GCGC) digesting the unmethylated fragments, ligation and ligation-digestion sample, respectively. Ligated probe sets are amplified by PCR

and subjected to capillary electrophoresis. By comparison of the ligated sample (indicative for the amount of total DNA, methylated as well as unmethylated, with the ligation-digestion sample (indicative for the amount of methylated DNA), the amount of methylation can be calculated. For methylation analysis, probe mixes ME001 and ME002 were purchased from MRC-Holland (Amsterdam, the Netherlands). The probe mix contains 25 probe sequences of which 15 sequences (control probes) are not influenced by *HhaI* digestion. All MLPA probe pairs code for unique human single copy DNA sequences and were designed and prepared as described by Schouten *et al.* [33]. Probe sequences, gene loci and chromosome locations can be found at www.mlpa.com. MLPA was performed as described by the manufacturer with minor modifications. In short, DNA (100-200 ng) was dissolved in 5 µl TE-buffer (10 mM Tris pH 8.2, 1 mM EDTA pH 8.0), denatured and subsequently cooled down to 25° C. After adding the probe mix, the sample was denatured and the probes were allowed to hybridise (16 hrs at 60° C). Subsequently, the samples were divided in two and one half of the samples was ligated, whereas for the other part of the samples ligation was combined with the *HhaI* digestion enzyme. This digestion resulted in ligation of only the methylated sequences. PCR was performed on both parts of the samples in a volume of 50 µl containing 10 µl of the ligation reaction mixture using the PTC 200 thermal cycler (MJ Research Inc., Waltham, USA) 33 cycles of denaturation at 95° C for 20 secs, annealing at 60° C for 30 secs and extension at 72° C for 1 min with a final extension of 20 mins at 72° C. An additional agarose gel electrophoresis was used to check MLPA efficiency [34]. Aliquots of 1 µl of the PCR reaction were combined with 0.3 µl LIZ-labeled internal size standard (Applied Biosystems, Foster City, USA) and 8.7 µl deionised formamide. After denaturation, fragments were separated and quantified by electrophoresis on an ABI 3730 capillary sequencer and Genemapper analysis (both Applied Biosystems). Peak identification was checked visually and values corresponding to peak size in base pairs (bp) and peak heights were used for further data processing. Instead of peak height, peak area can also be used [25]. The validity of the probes was checked by the analysis of normal DNA [43]. Furthermore, the sensitivity of ME001 and ME002 was established in a titration experiment in which normal DNA isolated from lymphocytes, which then was methylated *in vitro* using SSSI (New England

Biolabs, Ipswich, USA) as described by the manufacturer. Methylated samples were diluted to 75% methylated (M), 50% M and 25% M using the original unmethylated DNA [43]. Data analysis was performed in Excel as described by the manufacturer of the MLPA kits. First, the fraction of each peak is calculated by dividing the peak value of each probe amplification product by the combined value of the control probes within the sample, this in order to compensate for differences in PCR efficiency of the individual samples. For hypermethylation analysis this 'relative peak value' or so-called 'probe fraction' of the ligation-digestion sample is divided by the 'relative peak value' of the corresponding ligation sample, resulting in a so-called 'methylation-ratio' (M-ratio). Aberrant methylation was scored when the calculated M-ratio was > 0.25. The ME001 kit included the following genes: *PTEN*, *CD44*, *GSTP1*, *ATM, IGSF4, CDKN1B, CHFR, BRCA2, CDH13, HIC1, BRCA1, TP73, TIMP3, CASP8, FHIT, MLH1* (2 probes, *MLH1a and MLH1 b), RASSF* (2 probes, *RASSF1a and RASSF1b), RARB, VHL, APC, ESR1, CDKN2A, CDKN2B,* and *DAPK1.* And the ME002 kit included the following genes: *PTEN, MGMT* (2 probes*, MGMT-a* and *MGMT-b), CD44, WT1, GSTP1, ATM-a, IGSF4-a, STK11, CHFR, BRCA2, RB1* (2 probes, *RB1-a* and *RB1-b), THBS1, ASC, CDH13, TP53, BRCA1, TP73, GATA5, RARB, VHL, ESR1, PAX5A, CDKN2A*, and *PAX6.*

Statistical analysis

The Wilcoxon-Mann-Whitney test was used to compare the age of the patients and changes in copy numbers or number of hypermethylated genes in tumours of patients with FAP vs. those of patients with sporadic cancer. Fisher's exact test was used to examine differences between FAP-related and sporadic carcinomas in the frequencies of chromosomal copy number changes and percentage carcinomas showing methylation. The chi-square test was used for the association between copy numbers or methylation and histopathological characteristics. $P < 0.05$ was considered to be statistically significant (SPSS 12.0.1 for Windows 2003, SPSS Inc, Chicago, USA).

Results

Histological evaluation of the FAP-related tumours

The histopathological characteristics of the five FAP-related and ten sporadic carcinomas are summarised in Table 1. All FAP-related carcinomas were adenocarcinomas with moderate-to-poor differentiation. One carcinoma was located in the ampullary region. Three carcinomas showed a circumscriptive growth pattern and two carcinomas a diffuse growth pattern. There was none to little peritumoural inflammation in four FAP-related carcinomas. Moderate-to-extensive intratumoural inflammation was observed in two carcinomas and fibroblastic reaction was present in three carcinomas. In the majority of carcinomas invasion through the bowel wall was present (stage T3/T4). In three patients, lymph node metastases were present.

Histological evaluation of the sporadic tumours

Three carcinomas were located in the ampullary region. One sporadic tumour was a mucinous carcinoma. The adenocarcinomas were poorly (5/10), moderately (2/10) and well (2/10) differentiated. Circumscribed and diffuse growing carcinomas were observed in five and four of the patients, respectively. Peritumoural as well as intratumoural inflammation was moderate-to-extensive in four carcinomas. A fibroblastic reaction was present in nine carcinomas at a moderate-to-extensive rate. Most sporadic carcinomas (9/10) were in an advanced stage (T3/T4) and in three patients, lymph node metastases were observed. No differences in histopathological characteristics were observed between the FAP-related and sporadic carcinomas.

Table 1: *Histological characteristics of sporadic and familial adenomatous polyposis-related tumours.*

ID	Sex	Age	Type	Differentiation	Growth pattern	Peritumoural inflammation	Intratumoural inflammation	Fibroblastic reaction	T	N	Tumour diameter (cm)	Ampullary region
F1	F	50	Adeno	Poor / Moderate	Circumscript	+/-	+/-	+/-	T4	N1		Yes
F2	M	58	Adeno	Moderate	Circumscript	+/-	++	+	T3	N1	12 cm	No
F3	M	41	Adeno	Poor	Diffuse	-	-	+/-	T4	N0		No
F4	M	55	Adeno	Moderate	Circumscript	+	+	+/-	T1	NX		No
F5	M	65	Adeno	Poor	Diffuse	+/-	+/-	++	T3	N1	> 2 cm	No
S1	M	78	Adeno	Poor	Diffuse	+/-	+/-	+	T2	N0	5 cm	No
S2	M	69	Muc		Circumscript	+/-	+/-	+/-	T4	N0	2	No
S3	F	76	Adeno	Poor	Diffuse	+/-	+/-	++	T4	N1		No
S4	M	73	Adeno	Poor	Budding	+	+/-	+	T4	N1	4	No
S5	M	79	Adeno	Moderate	Circumscript	+/-	+/-	+	T4	N0	13	No
S6	M	60	Adeno	Moderate	Circumscript	++	+/-	+	T3	N0		Yes
S7	M	25	Adeno	Poor	Circumscript	+	+	+	T3	N0	9.5	Yes
S8	M	32	Adeno	Poor	Diffuse	+	+	++	T3	N0	5	No
S9	F	72	Adeno	Well	Diffuse	+/-	+/-	+	T4	N1	5	Yes
S10	F	68	Adeno	Well	Circumscript	+/-	+/-	++	T3	N0	7.5	No

ID: F, FAP-related tumour; S, sporadic tumour. Sex: F, female; M, male. Type: Adeno, adenocarcinoma; Muc, mucinous carcinoma. Peritumoural inflammation, intratumoural inflammation and fibroblastic reaction: -, absent; +/-, little; +, moderate; ++, extensive

Comparative genomic hybridisation

Chromosomal imbalances were detected in the majority of the FAP-related carcinomas (4/5). The mean number of changes was 3.8 (range 0–9). The mean number of gains (2.2; range 0-4) did not differ from the DNA copy number losses (1.6; range 0-4). For the sporadic carcinomas, 9 out of 10 tumours showed DNA copy number changes with an average of 4.6 changes (range 0-10) per tumour. The number of chromosomal gains was significantly higher than the losses 3.4 (range, 0-8) versus 1.2 (range 0-5), respectively (p= 0.03). The chromosomal gains and losses are listed in Table 2 whereas Figure 1 shows a schematic presentation of the chromosomal imbalances detected in the FAP-related and sporadic carcinomas.

In the FAP-related carcinomas, chromosomal gains were detected in regions on chromosome 7 (n = 1), 8 (n = 2), 11 (n = 1), 14 (n = 1), 17 (n = 2), 18 (n = 1), 19 (n = 2) and 20 (n = 1) whereas losses were observed on chromosome 1 (n = 1), 4 (n = 1), 10 (n = 1), 11 (n = 1), 15 (n = 2), 17 (n = 1) and 18 (n = 1).

In the sporadic carcinomas, gains most frequently (30% of the tumours) involved chromosomes 8, 12, 13 and 20. For these chromosomes the common regions of overlap were 8q11-13, 8q24-qter, 12p, 12q11-q21, 13q, and 20q. Clear copy number gains (ratio > 1.4) involved 13q, 12p and 5p11-13. High copy number amplifications (ratio > 1.6) were seen on 8q24-qter and 20q. Genetic losses were observed in regions on chromosome 2 (n = 1), 6 (n = 2), 8 (n = 1), 9 (n = 2), 15 (n = 1), 17 (n = 2), 18 (n = 2) and 21 (n = 1).

A significant difference in chromosomal imbalances between the FAP-related and sporadic carcinomas was seen on chromosome 12. In 6/10 of the sporadic carcinomas, gains in regions of this chromosome were observed, whereas no gains were detected in the FAP-related carcinomas (p = 0.04). Also for chromosome 13, genetic gains (even a clear copy) were seen in the sporadic carcinomas but not in the carcinomas of patients with FAP (p = 0.17).

Figure 1: *Summary of all chromosomal imbalances detected by CGH in sporadic and familial adenomatous polyposis-related duodenal carcinomas. Lines on the left and right of the chromosomes indicate respectively losses (red) and gains (green). A thin line indicates genetic aberrations crossing the 0.8 or 1.2 threshold, while clear copy changes crossing the 0.6 and 1.4 are indicated by a thick line (dark green). High copy changes, indicated by a ratio lager than 1.6 are indicated by an additional spot on a line (dark green).*

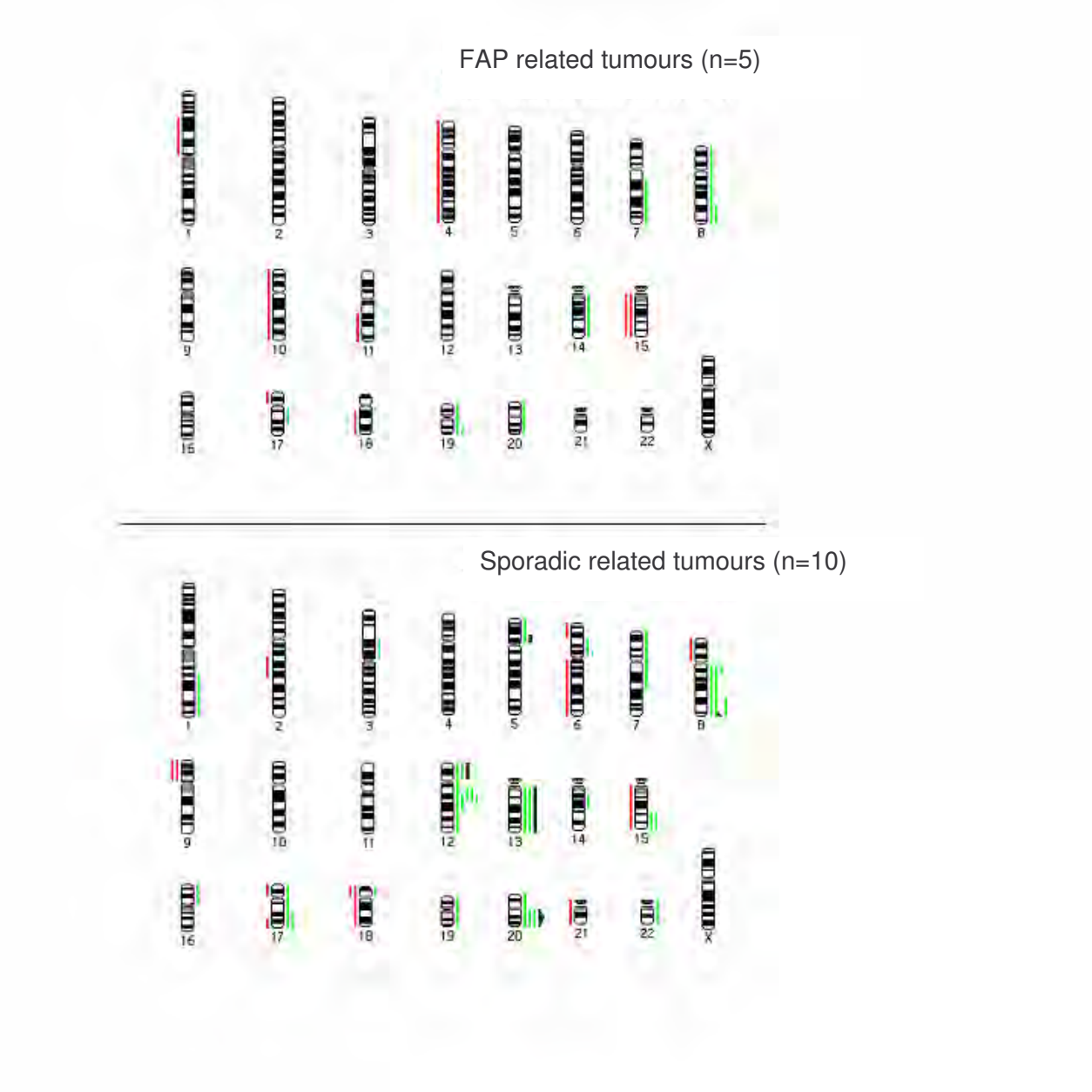


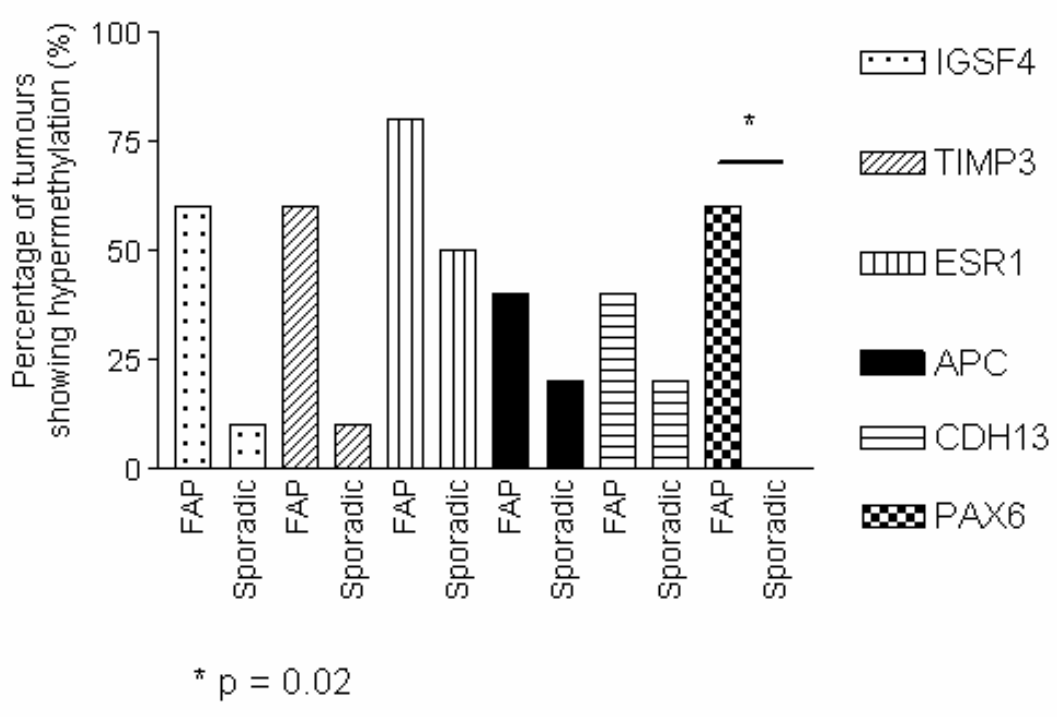
Table 2: *Chromosomal imbalances as identified by CGH present in sporadic and familial adenomatous polyposis-related tumours.*

ID	CGH gains	CGH losses	Number of changes
F1	7q21-qter, 14q, 17q11-23, 18q11-12	10q, 15q, 17p, 18q12-qter	8
F2	8q23-qter, 11q11-13, 17q11-21, 19, 20	1p11-32, 4, 11q14-qter, 15q	9
F3			0
F4	8		1
F5	19q32-qter		1
S1	6p11-12, 12q14-21, 13q, 15q24-qter	2q21-24, 21	6
S2	12q13-15, 20		2
S3	7pter-q22, 8q (8q24-qter high copy), 13q (clear copy), 22	8p, 9p, 15q, 17p, 18	9
S4	8q, 13q	9p, 17q23-qter	4
S5			0
S6	1q23-qter, 5p, 16p, 19, 20q		5
S7	12p (clear copy), 17	18p	3
S8	12		1
S9	3p11-14, 6p11-21, 12q13-15, 12p, 14q12-21, 15q24-qter, 18p, 20q (high copy)	6p21-pter, 6q	10
S10	8q11-13, 8q23-qter, 5p11-13 (clear copy), 12q14-15. 17q22-qter, 20q		6

Methylation–specific multiplex ligation-dependent probe amplification

For both immunoglobulin superfamily genes member 4 (*IGSF4*) and TIMP metallopeptidase inhibitor 3 (*TIMP3*), hypermethylation was found in 3/5 FAP-related carcinomas and 1/10 of the sporadic carcinomas (p = 0.08). Estrogen receptor 1 (*ESR1*) showed hypermethylation in four FAP-related carcinomas and in five sporadic carcinomas (p = 0.29). Two FAP-related carcinomas and two sporadic carcinomas showed hypermethylation in the adenomatous polysis coli (*APC*) and in H-cadherin (*CDH13*) genes (p = 0.41). A significant difference was observed between the FAP-related and sporadic carcinomas for the methylation of the paired box gene 6 (*PAX6).* Hypermethylation of *PAX6* was observed in 3/5 FAP-related carcinomas versus no hypermethylation in the sporadic carcinomas (p=0.02) (see Figure 2). Hypermethylation of glutathione S-transferase Pi (*GSTP*) and mutL homolog 1 (*MLH1*) was observed in one sporadic tumour. In one FAP-related tumour, hypermethylation of checkpoint with forkhead and ring finger domains (*CHFR*) and retinoblastoma 1 (*RB1 b*) was seen. The average number of hypermethylated genes was 4 ± 3 in FAP-related carcinomas versus 1 ± 1 in the sporadic carcinomas (p= 0.08). The following probes performed less reliable when using DNA isolated from formalin-fixed and paraffin-embedded tissue and are therefore excluded from further analysis: *CDKN2B* (ME001), *MGMT, WT1, ASC, STK11, GATA5, ESR1, PAX5A* and *CHFR* (all from ME002).

Figure 2: *Percentage of sporadic and familial adenomatous polyposis-related duodenal carcinomas showing hypermethylation in the genes IGSF4, TIMP3, ESR1, APC, CDH13 and PAX6.*



Discussion

The genetic pathways leading to the development and progression of small bowel carcinomas are not well characterised. Sporadic carcinomas develop infrequently in the duodenum, while it is the main site for malignant extra-colonic manifestations in patients with FAP. This study compares chromosomal and methylation alterations in duodenal FAP-related and sporadic carcinomas. In order to reveal similarities or differences in the pathways leading to these cancers, the data is also compared to literature data on colorectal and gastric cancer.

We observed hypermethylation in several well-known tumour suppressor genes in the sporadic duodenal carcinomas. In accordance with the data in

our study, a similar frequency of hypermethylation of *TIMP3* (10%) was seen in colorectal carcinomas [34, 35]. In contrast, a higher frequency of hypermethylation was found in colorectal carcinomas for *APC* (± 50% vs. 20% in our study) [34, 35] and for *CDH13* (65% vs. 20% in our study) [36]. In addition, frequent hypermethylation of the genes *CDKN2A* [37, 38] and *RASSF1A* [39, 40] was found in colorectal tumours, in contrast, no hypermethylation was found in our study. Almost no hypermethylation was found in the promotor region of the *PAX6* gene in colorectal carcinomas [41]. For gastric cancer, the frequencies of hypermethylation of *APC* and *TIMP3* were higher compared to our findings (± 78% and ± 43%, respectively). Similar to colorectal cancer, *CDKN2A* and *RASSF1A* are also frequently hypermethylated in gastric cancer in contrast to the duodenal carcinomas studied here [42]. Although, the MS-MLPA and the conventional MS-PCR showed very similar results in our hands for the *MGMT* [43] gene, differences found in comparison with literature data may arise from the different methods.

Several genomic imbalances in duodenal carcinomas were detected by CGH. Similar to what has been described for colorectal carcinomas; sporadic duodenal carcinomas showed frequent gains on chromosomes 8q, 12p, 13q and 20q. In contrast to sporadic colorectal cancer, only one loss at 18q was observed [44-47] in the duodenal carcinomas. In gastric carcinomas, again frequent gains involved 8q, 13q and 20q but only occasionally 12p [48, 49]**.** In contrast to both colorectal and gastric carcinomas, sporadic duodenal carcinomas often showed gains in the region 12q13-21. Interestingly, a difference was observed between sporadic and FAP-related carcinomas with respect to copy changes on chromosomes 12 and 13 as detected by CGH. Gains were only present in the sporadic carcinomas and not in the FAP-related carcinomas. Chromosomal gains on chromosome 12p were also observed in pancreatic [50, 51] and gastric tumours [49]. Recently, gains of 12p were shown to be late events in liver metastases of colorectal carcinomas, indicating their role in tumour progression [46]. Likewise, gains of 12p were often observed in the advanced stages of a wide variety of tumours [46, 52, 53]. In the current study, most carcinomas also presented at an advanced stage. However, since the tumour stage was comparable in both groups, this could

not explain the difference detected between sporadic and FAP-related carcinomas. Possible candidate genes on chromosome 12 include *KRAS2*, *cyclinD2*, *MDM2* and *WNT1*. In addition, gains of 13q as observed here in sporadic duodenal tumours, were shown frequently in both colorectal [44, 45, 47, 54] and gastric carcinomas [48, 49]. Also a difference was observed in the hypermethylation status of the *PAX6* gene between sporadic and FAP-related duodenal carcinomas. PAX6 is a highly conserved transcription factor, which plays an important role in the normal embryological development [55]. Furthermore, this transcription factor is implicated in pancreatic and intestinal endocrine cell fate determination and in eye and brain development [56]. However, the role of PAX6 in the development of intestinal carcinomas is less clear and also the difference in hypermethylation of *PAX6* between FAP- and sporadic carcinomas is difficult to explain. Future research must further elucidate the exact role of PAX6 in the development of duodenal carcinomas.

The mutations in the *APC* gene (located on chromosome 5q21-22) often found in patients with FAP lead to a disturbed function of the APC protein. The key tumour suppressor function of APC is to regulate the stability and cellular localisation of β-catenin [57]. β-catenin is a bifunctional protein with a crucial role in cell-cell adhesion [58] and a signalling role in the Wnt pathway [59]. Loss of functional APC leads to a disturbed Wnt signalling pathway, which is involved in many types of cancer [60] and aberrant activation of this pathway could possibly result in chromosomal instability as found in colon cancer [61]. The genes of *WNT1* and *WNT10B*, both members of the Wnt gene family and encoding for Wnt signalling proteins, are located in the chromosome 12q13 region [62]. Interestingly, in three sporadic carcinomas gains are found in this region. Given the importance of Wnt signalling in tumour formation, this may suggest that the chromosomal imbalances on chromosome 12 in the sporadic carcinomas are associated with Wnt signalling abnormalities. One has to realise that Wnt signalling is already disturbed in FAP-related carcinomas, which may explain the absence of chromosomal aberrations in this region of chromosome 12 here. So although different aberrations are detected in FAP-related (*APC* mutations) and sporadic (+12q) carcinomas, their effects may be similar.

For FAP-related carcinomas, there are only karyotyping studies of colonic adenomas [63], colonic carcinomas [64] and desmoid tumours [65]. In comparison to CGH, a disadvantage of karyotyping is that it requires the culture of fresh tumour cells, which can only be achieved for some of the (malignant) tumours and which may introduce culture artifacts such as clonal selection [24]. Since macro-dissected tumour tissue was used in this study, almost only tumour DNA was used for the CGH, thus increasing the accuracy of the measurements. However, carcinomas used in this study were routinely processed, formalin-fixed and paraffin-embedded. DNA isolated from paraffin-embedded tissue is often degraded, with the bulk of the DNA showing a fragmented size. This could complicate the nick translation for the CGH and may lead to inferior results when compared to results obtained by CGH after using frozen tissues. Therefore, some chromosomal imbalances could have been left undetected in the paraffin-imbedded tumours investigated here, especially in the older specimens.

The karyotyping study of desmoid tumours in patients with FAP mainly showed a defect on chromosome 5q, where the *APC* gene is located. [65]. The FAP-related adenocarcinomas investigated here showed no abnormalities in this region. However, small deletions present in the *APC* gene could be undetected by CGH. Interestingly, we found hypermethylation of the *APC* promoter region in two FAP-related carcinomas, suggesting biallelic inactivation of the *APC* gene. To our knowledge, in the only study described so far which investigates a colon carcinoma of a patient with FAP, no abnormalities on chromosome 12 were observed and that is in accordance with our results [64]. Sporadic colon carcinomas show gains of 12p in approximately 30% of the cases [44].

Both sporadic and FAP-related carcinomas showed genetic gains on chromosome 8 (especially 8q). Diep *et al*. [46] suggested that gains of 8q could be involved in establishing distant colorectal metastases. Gains of 20q, which may be an early change in both primary colorectal carcinomas and their liver metastases, were also seen in sporadic as well as FAP-related carcinomas.

Furthermore, the frequency of gains of 20q was reported to increase with Dukes' stages, emphasising their role in tumour progression as well [46, 54]. In addition, frequent gains of 20q have been reported in other tumours, including gastric adenocarcinomas [66].

The current study showed no differences in chromosomal imbalances between tumours in the ampullary region and more distally located carcinomas, probably due to small numbers. In accordance with Chang *et al*. [21], several gains were found in sporadic tumours located in the ampullary region, such as on chromosomes 1q, 3p, 12p, 14q, 18p and 20q. In contrast to Blaker *et al.* [20], who reported frequent losses of 18q in sporadic small bowel adenocarcinomas, only one case of loss of chromosome 18 and one loss of chromosome 18p were observed in this study. The study of Blaker *et al.* [20] however, included only four duodenal carcinomas, of which two tumours showed loss of chromosome 18 (q). The two only studies describing hypermethylation of promoter regions of genes in small bowel carcinomas also showed both similarities and differences to our study. Brücher *et al*. [22] demonstrated the same frequency in hypermethylation of *APC*, however differences were observed for the prevalence of hypermethylation of *MLH1* and *CDKN2A.* These differences may have been caused by our smaller study population. In comparison with results of Kim *et al.* [23], the same high frequency of hypermethylation was found here for *EST1*, although *MLH1* and *CDKN2A* also differed from our study.

In summary, the chromosomal and methylation alterations of 15 duodenal carcinomas were analysed in this study. The results suggest that gains on chromosomes 8, 17 and 19, and losses on chromosome 15 might play a role in the development and/or progression of FAP-related carcinomas (n=5). In the sporadic carcinomas (n=10), frequent gains were seen on chromosomes 8, 12, 13 and 20. These findings are similar to what has been described for sporadic colorectal and gastric carcinomas. In contrast to results in sporadic colorectal cancer, only once was a loss of 18q observed in sporadic duodenal carcinomas. Furthermore, in contrast to both colorectal and gastric carcinomas, sporadic duodenal tumours often showed gains in the region

12q13-21. In addition, although the numbers of carcinomas studied here are small, different patterns of chromosomal imbalance could be detected in sporadic versus FAP-related carcinomas. Gains on chromosome 12 were not observed at all in duodenal carcinomas of patients with FAP, suggesting that identification of the genes in 12q13-21 could lead to a better understanding of the carcinogenesis pathways in both sporadic as well as FAP-related duodenal carcinomas. Interestingly, *APC* mutations present in patients with FAP may have a similar effect on the Wnt signalling as gains of 12q in sporadic tumours, suggesting that even though the aberrations detected may differ, a similar pathway is affected. Furthermore, methylation of multiple CpG-islands is present in both sporadic and FAP-related duodenal carcinomas whereas the methylation status of *PAX6* seems to be different in FAP-related carcinomas compared to sporadic carcinomas.

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Chapter 6

Ursodeoxycholic acid intervention in patients with familial adenomatous polyposis: a pilot study.

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Duodenal adenomas, especially those clustered in the peri-ampullary region, are the main pre-malignant extra-colonic manifestations in patients with familial adenomatous polyposis (FAP), which suggests a role of bile in this adenomatosis. Ursodeoxycholic acid (UDC), a bile acid with chemopreventive properties was tested in patients with FAP to find out whether UDC is able to reduce cytotoxicity or genotoxicity of duodenal bile or to reduce the cyclooxygenase-2 (COX-2) expression in the duodenum. A three-month unblinded intervention with UDC (up to 25 mg/kg body weight daily) was performed in 5 patients with FAP. Bile acids in duodenal bile were determined by gas chromatography. Cytotoxicity of bile was quantified by the WST-1 assay and genotoxicity was determined using the Comet assay. COX-2 expression in duodenal biopsies was determined immunohistochemically. This high dose of UDC was well tolerated and duodenal bile was highly enriched with UDC. Cytotoxicity of the heat inactivated post intervention bile was 2-fold lower compared to the pre-intervention bile ($p = 0.043$) and no difference in genotoxicity was observed. Duodenal COX-2 expression was non-significantly reduced ($p = 0.125$) after the intervention. UDC enrichment of bile in patients with FAP did lower the cytotoxicity of heat inactivated bile whereas duodenal COX-2 expression was non-significantly reduced. The results of this small pilot study warrant a more extensive study on chemoprevention with UDC in patients with FAP.

Introduction

Familial adenomatous polyposis (FAP), caused by mutations in the *Adenomatous Polyposis Coli* (*APC*) gene, is characterised by the early onset of hundreds to thousands of adenomas throughout the large bowel [1, 2]. Left untreated, there is nearly 100% progression to colorectal cancer by the age of 35-40 years. Prophylactic colectomy is the recommended treatment to eliminate the risk of colorectal cancer. In addition, patients with FAP run the risk of developing extra-colonic manifestations and the duodenum is the main site for (pre-)malignant development [3, 4]. The prevalence of the mainly peri-ampullary located adenomas varies from 50% to over 90% [4-6]. With a prevalence of 2-5%, duodenal carcinomas are now, together with desmoid tumours, the leading causes of cancer-related mortality in patients with FAP [5, 7-10]. Since both incidence and severity of the duodenal adenomatosis increases with age, and since the FAP population is aging, the problems with respect to the development of duodenal adenomas and malignancies will further increase [9].

The clustering of the duodenal adenomas around the Ampulla of Vater suggests that bile plays a role in the formation and/or progression of these adenomas. In the liver, the primary bile acids, cholic acid and chenodeoxycholic acid, are synthesised and conjugated with taurine or glycine. The conjugated bile acids are excreted into the gall bladder and released into the small intestine after stimulation of the gall bladder. Bile acids are re-absorbed into the terminal ileum and only 5 per cent of the bile acid pool enters the large intestine. Bacteria in the large intestine deconjugate the bile acids and are responsible for the 7-α dehydroxylation of the primary bile acids into the secondary bile acids, deoxycholic acid and lithocholic acid from cholic acid and chenodeoxycholic acid, respectively. The circulating bile acid pool in healthy individuals is composed of about 35% cholic acid, 35% chenodeoxycholic acid, 25% deoxycholic acid and less than 5% lithocholic acid [11, 12]. After colectomy however, the content of secondary bile acids in the circulating bile acid pool is significantly reduced in patients with FAP as well as in non-FAP patients [13]. The secondary bile acids especially are known tumour promoters in the gastrointestinal tract [14-17]. In contrast, ursodeoxycholic acid

(UDC), a tertiary bile acid hardly present in human bile, may act as a chemopreventive drug in patients at risk of colon cancer, as suggested by several pre-clinical studies [18, 19]. Wali *et al.* [19] found an inhibiting effect of UDC on the induction of cyclooxygenase-2 (COX-2), a key enzyme in the regulation of cell proliferation, in an animal model of colonic carcinogenesis. More importantly, in patients with ulcerative colitis or primary sclerosing cholangitis, the risk of colorectal dysplasia or cancer was reduced by UDC [20]. UDC treatment was also associated with a significant reduction in the recurrence of colorectal adenomas with high grade dysplasia in a large phase III clinical trial [21]. Furthermore, a combination of UDC and sulindac was proven effective in the prevention of intestinal adenomas in a mouse model of FAP [22].

Until now, the treatment of duodenal adenomas in FAP has been restricted to surgical intervention, with concomitant high morbidity and mortality [23] and therefore, new therapies, such as chemoprevention, would be preferable.

This pilot study explores the possibility of ursodeoxycholic acid as a chemopreventive agent for duodenal adenomas/carcinomas in patients with FAP. We therefore performed a pilot intervention study with ursodeoxycholic acid in five patients with FAP to find out if ursodeoxycholic acid is tolerated. In addition, we studied the effects on bile acid composition, cytotoxicity and genotoxicity of duodenal bile, sampled before and after the intervention. We also studied whether the intervention influenced the duodenal COX-2 expression levels.

Materials & Methods

Patients

In this study, 5 patients (average age 42, range 33–50 years; 4 males / 1 female) with FAP were included. Three patients had undergone sub-colectomy with an ileorectal anastomosis and the other two patients had a colectomy with an ileostomy. The patients had no history of liver disease, pancreatic disease or gall stones. Informed consent was given by the patients and the study was approved by the local medical ethical review committee.

Study design

A three-month unblinded intervention with ursodeoxycholic acid was performed in 5 patients with FAP. Ursodeoxycholic acid was administered initially at 300 mg per day during the first week. During the second week, the dose was gradually increased to 600 mg per day, followed by two weeks at 1200 mg per day. During weeks 5 –12, the patients received a daily dose of 25 mg/kg body weight [24]. After intravenous administration of 5 µg CCK-8 (Calbiochem-Novabiochem AG, Läufelfingen, Switzerland), duodenal bile and biopsies were collected twice during duodenoscopy with an Olympus TJF-160R side-viewing endoscope; once before the start of the intervention and once one day after the end of the ursodeoxycholic acid intervention period.

Bile acid analysis

Composition and concentration of bile acids in the duodenal bile of the patients with FAP, obtained both before and after the intervention, were determined by gas chromatography essentially as previously described [25, 26] with some minor modifications. In short, after enzymatic hydrolysis of duodenal bile with cholylglycine hydrolase, total deconjugated bile acids were extracted with C-18 bonded silica cartridges. After hydrolysis of esterified bile acids with 1 M KOH (60° C for 2 hrs), the unconjugated bile acids were separated from the neutral sterols by Lipidex DEAP chromatography and after methylation and silylation, bile acids were measured by capillary gas chromatography (Chrompack, CP9001 with column CP Sil 5CB, Varian Inc, Palo Alto, USA).

Pancreatic enzymes

To investigate the effect of the pancreatic enzymes on the cytotoxicity and genotoxicity assays, the duodenal bile was heat inactivated at 60° C for 30 mins.

Pancreatic enzyme activities

The enzymatic assay of trypsin in the duodenal bile was performed by photospectrometry with 0.25 mM Nα-benzoyl-L-arginine ethyl ester as a substrate, according to the instructions of the manufacturer (Enzymatic assay of trypsin, Sigma, St Louis, USA). Amylase activity in duodenal bile was

determined by the routine Clinical Chemistry Laboratory, Radboud University Nijmegen Medical Centre, the Netherlands.

Cell culture

Two cell lines were used to quantify cytotoxicity or genotoxicity in this study: HT-29 (colonic adenocarcinoma cells; ATCC HTB-40) and LT97 (colonic adenoma cell line derived from a patient with FAP [27]). Both cell lines were cultured in a humidified incubator (5% CO_2) at 37˚C using the serum-free PC-1 medium supplemented with 2 mM L-glutamine and a supplement provided by the manufacturer (Cambrex, Verviers, Belgium). The medium was changed every two days until cells reached 80% - 100% confluence.

Cytotoxicity measurements

After reaching confluence, the cells were seeded at a density of 15,000 cells /well in a flat-bottomed 96 wells plate (Costar, Corning Incorporated, Corning, NY, USA). The HT-29 cells were allowed to grow for 24 hrs, whereas the LT97 cells needed 48 hrs with an additional medium change to form a cell monolayer in these plates. The medium was removed and the cells were incubated with 200 µl test sample and controls. The PC-1 medium alone was used as a negative control and the PC-1 medium containing 3.2 mM unconjugated deoxycholic acid (Sigma, St Louis, USA) was used as a positive control for cytotoxicity. Wells without cells served as background correction for the measurements. Duodenal bile was diluted 20 times with PC-1 medium, sterilized by filtration on a low protein binding 0.2 µm filter (Acrodisc, Gelman sciences, Michigan, USA), and added to the wells in the following dilutions: 20, 40, 80, etc.until 20,480 times. Every dilution was measured in octuple. After incubating for 24 hrs, the medium was removed and new PC-1 medium containing 10% (v/v) proliferation reagent WST-1 (Roche, Penzber, Germany) was added to each well. In viable cells, WST-1 will be converted to formazan by mitochondrial activity. After two hours, the color intensity of the formazan formed was quantified in a microplate reader (Thermomax, Molecular Devices, Sopachem B.V., Wageningen, the Netherlands). The absorbance was measured against the background controls at a wavelength of 405 nm and a reference wavelength of 620 nm. The cell viability was expressed as the

percentage of absorption of test samples, compared with that of wells incubated with medium alone. Results are given as the dilution factors of the duodenal bile at which 50% of the cells survive. The mean value of a duplo experiment was used to calculate the overall mean of four independently reproduced experiments for each patient. For the group of patients, the results are given as the median with 25^{th} and 75^{th} percentiles.

Genotoxicity measurements

Genotoxicity was measured using the Comet assay. Since ursodeoxycholic acid enriched bile showed an effect on the cytotoxicity assay with the HT-29 cells, this cell line was also used in the Comet assay. To exclude the role of pancreatic enzymes and to underline the possible effect of ursodeoxycholic acid enriched bile, the genotoxicity of heat inactivated duodenal bile was measured. After harvesting the cells, cell suspensions of $2x10^5$ cells/ml were incubated with 10% (v/v) 0.1 mM hydrogen peroxide (positive control), phosphate-buffered saline (PBS) (negative control) or heat inactivated diluted duodenal bile for 30 mins at 37˚ C. The dilution of duodenal bile at which more than 90% cell viability was achieved in the cytotoxicity assay, was used in the comet assay. The cell pellets of 50 µl of the incubated cell suspensions were distributed with 75 µl of low melting point agarose on Comet slides (Trevigen Inc., Gaithersburg, USA), according to the instructions of the manufacturer. The slides were kept at 4˚ C for 30 mins and subsequently at 4 ˚ C for 60 mins in lysis buffer. Next, all the slides were placed in an electrophorese chamber (Owl Separation Systems, Portsmouth, USA) with alkaline buffer (1 mM EDTA, 300mM NaOH, pH 13) at 4 ˚C. After 30 mins, the current was switched on and electrophoresis was carried out at 25 V and 300 mA for 30 mins with the slides just covered. The slides were removed from the alkaline solution and washed three times for 5 mins at 4˚ C in neutralisation buffer (0.4 M Tris/ HCl, pH 7.5). Finally, the slides were placed in 100% ethanol for 10 mins at 4˚ C. The slides were allowed to air-dry overnight and were stored at 4˚ C until further analysis. All handling of the samples was performed under red light. The slides were stained with SYBR Green (2 µg/ml; Trevigen Inc.) and evaluation was performed by microscopic analyses with Kinetic Imaging Komet software 5.5 (Kinetic Imaging, Nottingham, UK). Fifty cells per slide were evaluated and the

percentage of fluorescence in the tail (TI, "tail intensity") was scored. The mean value of a duplo experiment was used to calculate the overall mean of two independently performed experiments for each patient. For the groups, results are given as the median with 25th and 75th percentiles.

Immunohistochemistry

The paraffin–embedded biopsies were cut into 4 μm sections. These sections were de-paraffinised in xylene, rehydrated and incubated with 3% hydrogen peroxide/PBS to block endogenous peroxidase. Antigen retrieval was performed using microwave exposure for 10 mins in 10 mM citrate buffer (pH 6.0). The slides were allowed to cool down for at least 90 mins. After pre-incubation with 20% normal horse serum, sections were incubated overnight at 4˚ C with 1:200 diluted primary antibody against COX-2 (Cayman Chemical Company, Michigan USA) in PBS/1% Bovine Serum Albumin (BSA), followed by the incubations with a biotinylated secondary antibody and avidin-biotin-peroxidase complex (Vector Laboratories Inc, Burlingame, USA) respectively. Finally, 3,3'-diaminobenzidene (DAB) was used as chromagen with haematoxylin counterstaining. Negative controls were constructed by omitting the primary antibody. The scoring of COX-2 staining was performed independently and in a blind manner by two of the authors (M.B and M.J.E.M.G). The following scoring criteria of the biopsies were formulated before the start of the analyses: 0, no staining; 1, weak cytoplasmatic and membranous staining (may contain strong staining in <10% of the cells); 2, moderate-to-strong staining in 10–90% of the cells; and 3, strong staining in >90% of the cells [28]. For the statistical analysis, scores 0-2 were categorised as "low expression" of COX-2 and score 3 as "high expression" of COX-2, see below.

Statistical analysis

The Wilcoxon matched-pairs signed-ranks test was used to examine differences in bile acid composition, cytotoxicity and genotoxicity of the duodenal bile, obtained before and after the intervention. The Mann-Whitney test was used for comparison of two unrelated parameters. The duodenal staining of COX-2 before and after the intervention was compared with the

McNemar test. P<0.05 was considered statistically significant. (SSPS 12.0.1 for Windows 2003, SPSS Inc., Chicago, USA).

Results

Patients

The high dose of ursodeoxycholic acid was tolerated well by four patients. One patient, taking the maximum dose, suffered from diarrhea and needed a dose reduction for a short period of time. After two days, this patient was able to continue with ursodeoxycholic acid at the dose of 25 mg/ kg body weight. No other side effects were reported by the patients.

Bile acid composition of duodenal bile

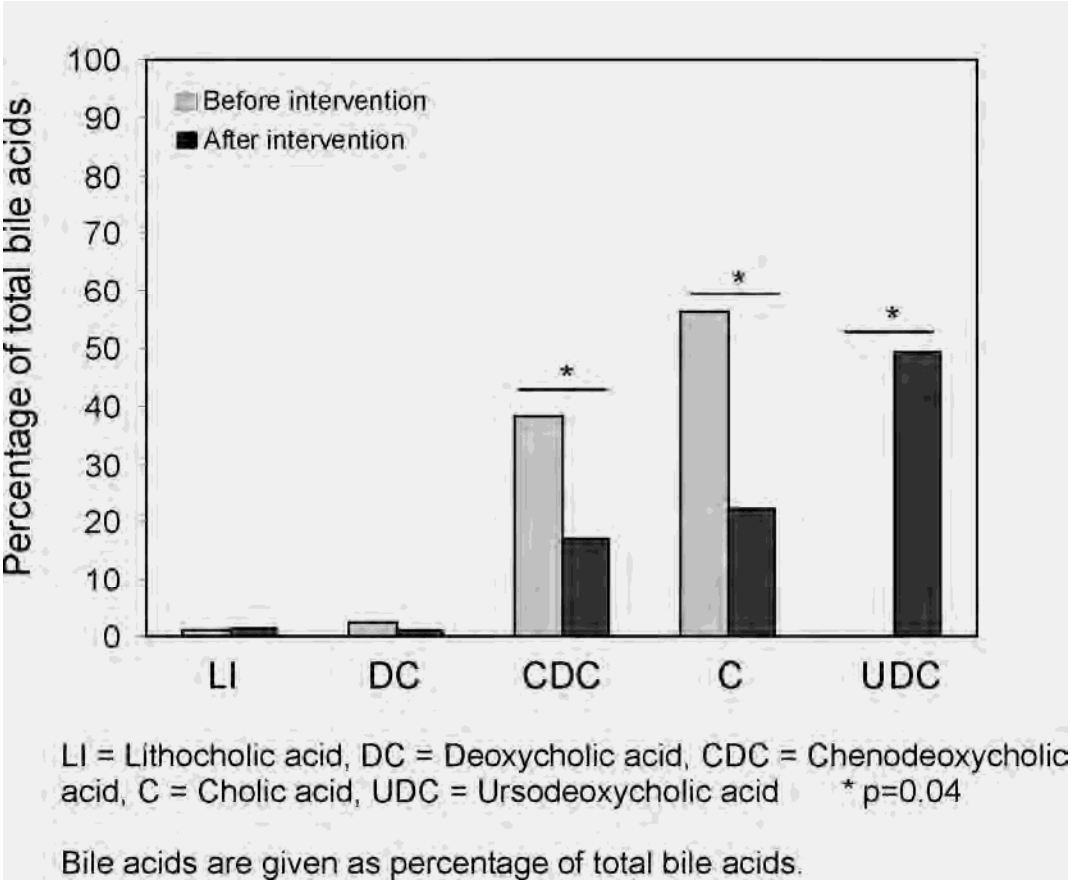
Figure 1 shows the bile acid composition of the duodenal bile of the patients with FAP, both before and after the intervention with ursodeoxycholic acid. The contents of the secondary bile acids, lithocholic acid and deoxycholic acid, were very low and did not differ before or after the intervention, whereas the levels of chenodeoxycholic acid and cholic acid were significantly lower after the intervention period ($p = 0.043$). The content of ursodeoxycholic acid was raised significantly from almost zero to 49% after the intervention period ($p = 0.043$). The total pre-intervention bile acid concentration decreased from 26.3 mmol/L to a post-intervention concentration of 8.4 mmol /L ($p = 0.080$), which correlated with lowered concentrations of bilirubin in the post-intervention duodenal bile ($p < 0.0001$).

Pancreatic enzymes

No differences were observed in the trypsin enzyme activity of duodenal bile before and after the intervention (48 (range 22-182) vs. 64 (range 27–254) units/mg protein, $p = 0.500$). After heat inactivation, the trypsin activity was significantly lower in duodenal bile obtained before and after intervention (both 1 unit/mg protein, $p < 0.001$).

Amylase activity showed no difference in bile before and after the intervention (269,600 (range 5600-299,800) vs. 241,140 range (12,340- 527,000) units/L, p = 0.500). After heat inactivation, the amylase activity was not significantly lower, either before or after the intervention (72,400 vs. 109,300 units/L, p= 0.210).

Figure 1: *Bile acid composition of duodenal bile from patients with FAP before and after ursodeoxycholic acid intervention.*



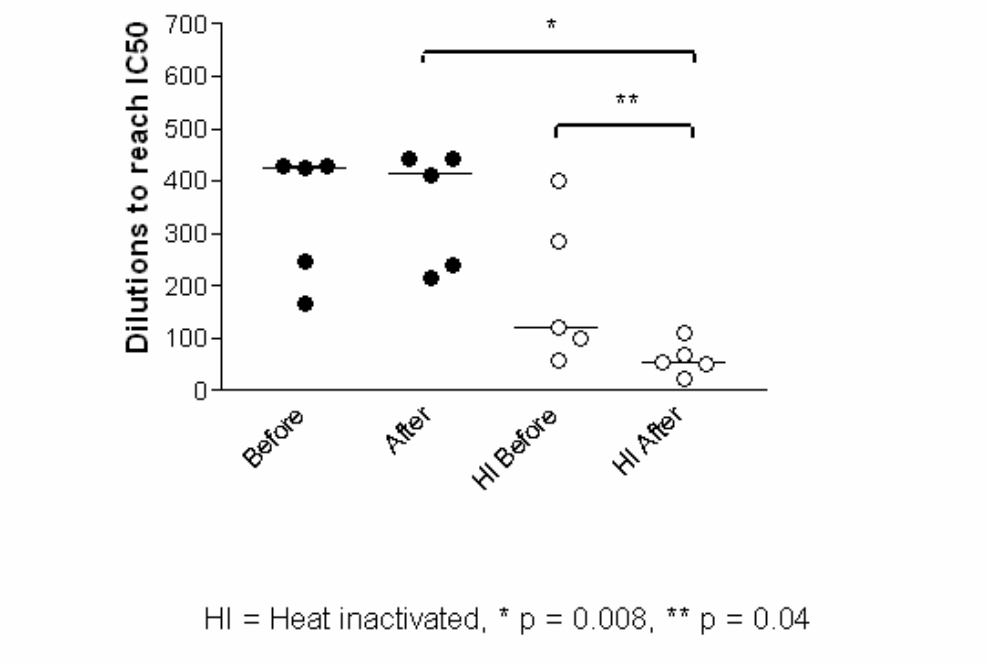
Cytotoxicity of bile

The cytotoxicity of duodenal bile obtained both before and after the ursodeoxycholic intervention period, measured by using the cell lines HT-29 and LT97 was not different (see Figures 2 & 3).

After heat inactivation, the cytotoxicity of the ursodeoxycholic acid enriched duodenal bile towards the HT-29 cells was approximately 2-fold lower when compared to the pre-intervention bile (median IC50 of 56 vs. IC50 of 121, p =

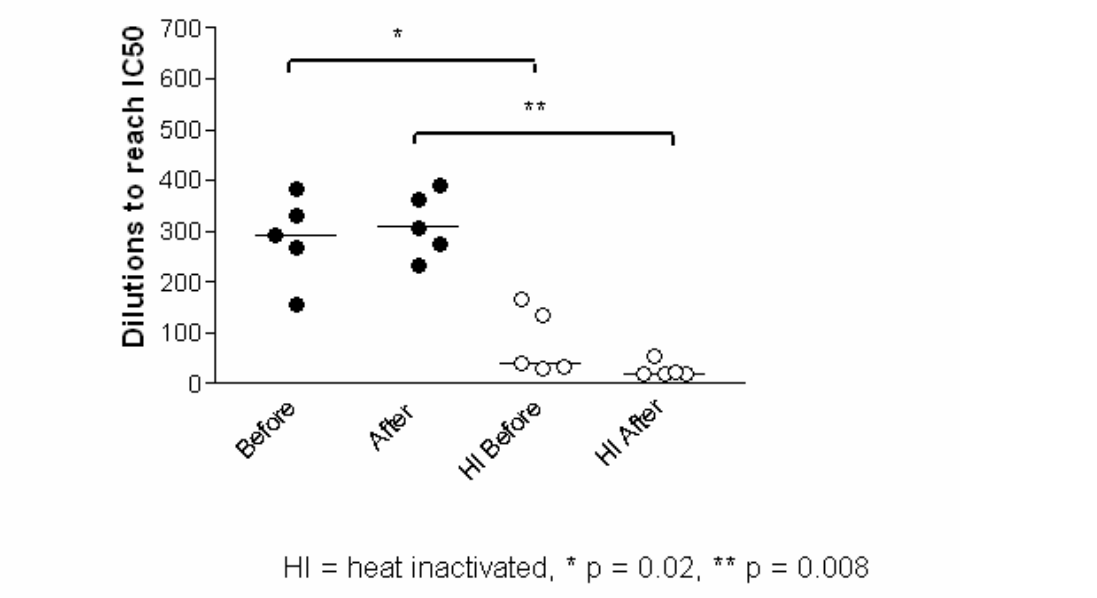
0.043). Furthermore, the duodenal bile became less cytotoxic compared with the non-heat inactivated duodenal bile (see Figure 2). In the HT-29 cell test system, the cytotoxicity of the heat inactivated duodenal bile was approximately 7-fold lower after the intervention (median IC50 of 56, p = 0.008) compared with the non-heat inactivated bile (IC50 of 413) (see Figure 2). Using the LT97 cells, after heat inactivation the cytotoxicity of post-intervention bile was 15 times lower than the same bile before heat inactivation (median IC50 of 21 vs. IC50 of 308, p = 0.008p = 0.008) (see Figure 3).

Figure 2: *Cytotoxicity: scatter plots showing the dilutions of (heat inactivated) duodenal bile at which 50% of HT-29 cells survive (IC50), obtained before and after the ursodeoxycholic acid intervention.*



HI = Heat inactivated, * p = 0.008, ** p = 0.04

Figure 3: *Cytotoxicity: scatter plots showing the dilutions of (heat inactivated) duodenal bile at which 50% of LT97 cells survive, obtained before and after the ursodeoxycholic acid intervention.*

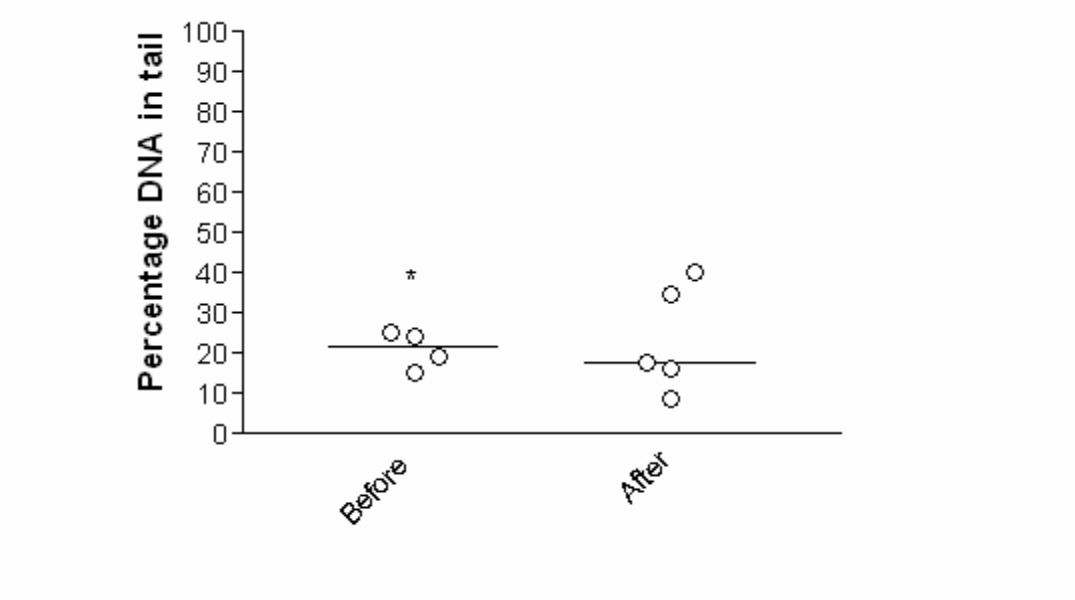


HI = heat inactivated, * p = 0.02, ** p = 0.008

Genotoxicity of bile

There was no difference in the percentage of DNA in the tails of HT-29 cells treated with PBS or with duodenal bile before intervention (17.5% vs. 21.9%, respectively; p = 0.286) nor with the ursodeoxycholic acid enriched duodenal bile obtained after the intervention period (17.5% vs. 17.7%, respectively; p = 0.841). No differences were observed in tail intensity after treatment with duodenal bile obtained before and after the intervention (21.9% vs. 17.7%, respectively; p = 0.715) (see Figure 4). There was a significantly higher amount of DNA in the tails of HT-29 cells incubated with hydrogen peroxide (positive control) compared with PBS (negative control) treated cells (89.6% vs. 17.5%, respectively; p = 0.009), demonstrating that the Comet assay is functioning properly.

Figure 4: *Genotoxicity:* s*catter plots showing the percentages of DNA in the tails of HT-29 cells, treated with heat inactivated duodenal bile, obtained before and after the ursodeoxycholic acid intervention.*



* Because of the unsuccesful Comet assay, the data of one patient was not available

Immunohistochemistry

Typical examples of immunohistochemical staining of COX-2 before and after the intervention are depicted in Figure 5. Granular cytoplasmatic and membranous staining of COX-2 was observed in all normal duodenal specimens. Before the intervention, four of the five patients with FAP showed strong duodenal staining of COX-2 in more than 90% of the cells. After the intervention, all five patients showed moderate-to-strong staining in 10-90% of the cells (see Figure 6, $p = 0.125$).

Figure 5: *Immunohistochemical staining for COX-2 in normal duodenal mucosa of patients with FAP, sampled before and after the intervention with ursodeoxycholic acid. A, Before intervention (original magnification X 200). B, After intervention (original magnification X 200). C, Before intervention (original magnification X 400). D, After intervention (original magnification X 400).*

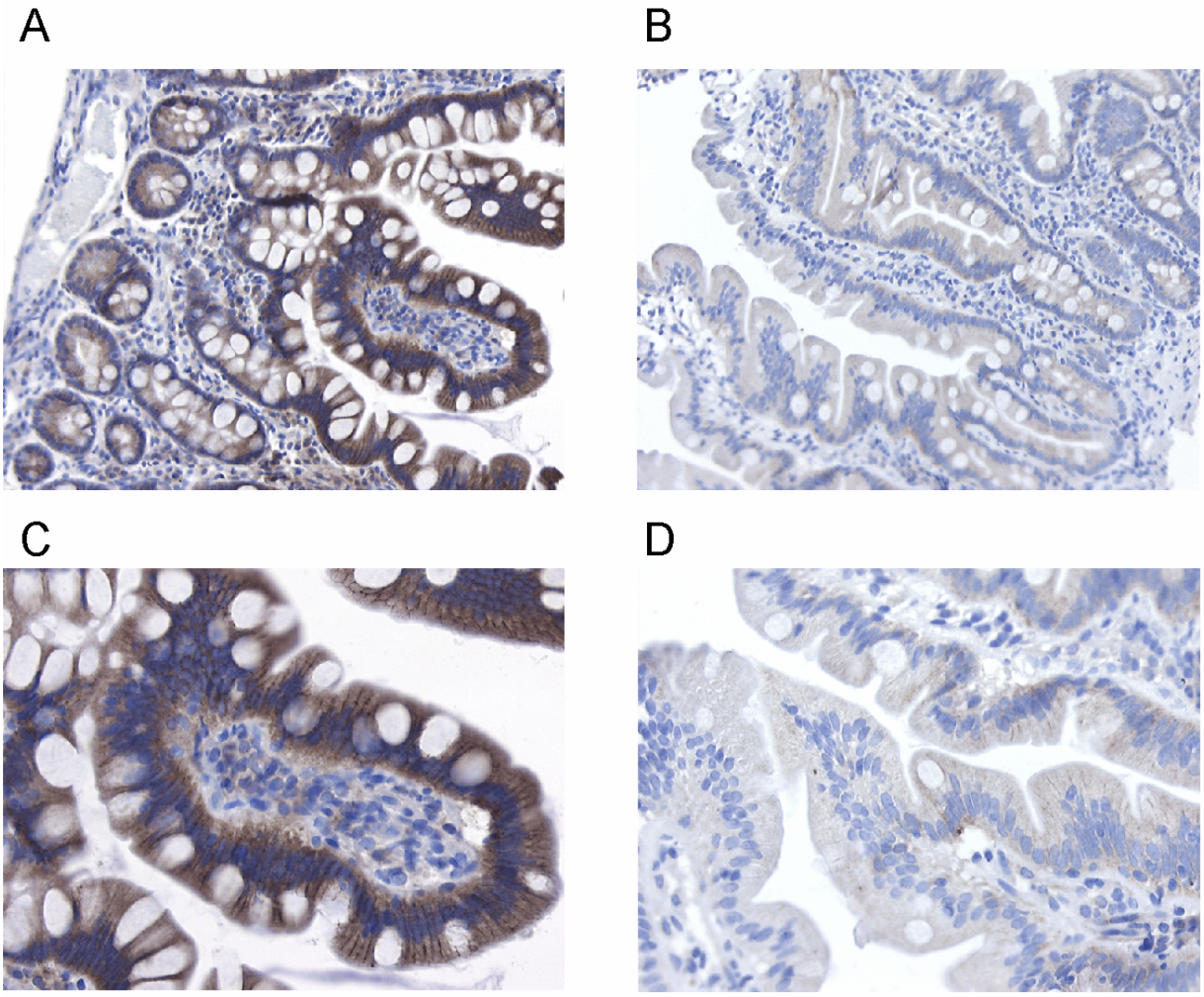
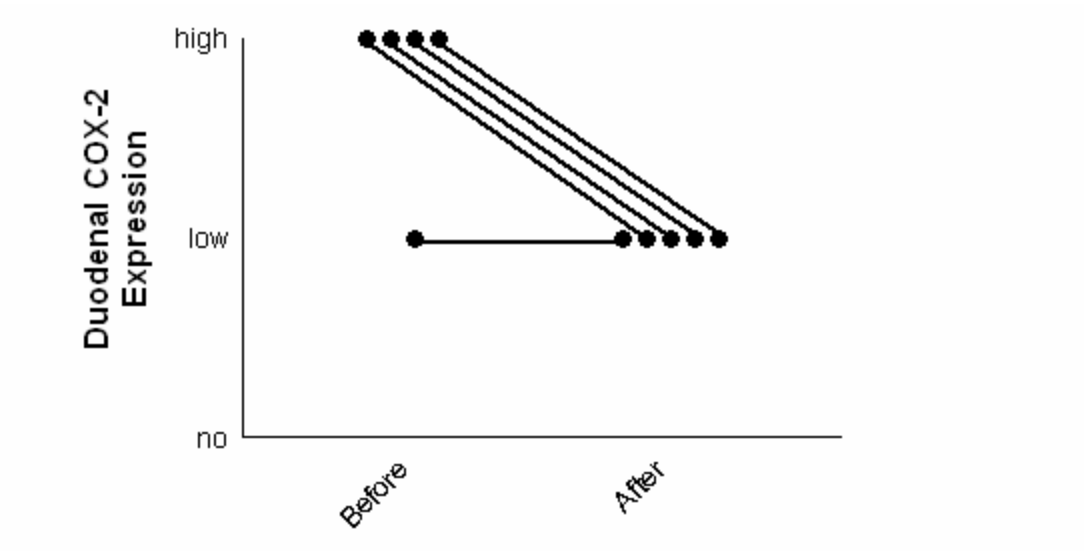


Figure 6: *Immunohistochemistry: scatter plots showing the duodenal COX-2 expression before and after the ursodeoxycholic acid intervention*



Discussion

Duodenal carcinomas and desmoid tumors are now the leading causes of cancer–related mortality in patients with FAP. Therefore the treatment of duodenal adenomas in patients with FAP remains a clinical challenge [5, 7-10]. Surgical intervention is associated with high morbidity and mortality [23]. Consequently, chemoprevention of adenomas and carcinomas would be highly desirable. This pilot study explores the possibility of using ursodeoxycholic acid as an agent to prevent duodenal adenomatosis and subsequent carcinogenesis in patients with FAP.

This study shows that ursodeoxycholic acid is tolerated well by FAP patients whose colon has been removed. Moreover, the duodenal bile is highly enriched with this tertiary bile acid. The amount of ursodexycholic acid in the duodenal bile after the intervention as found in this study (up to 50%) corresponds to the enrichment of up to 41%, as reported in the duodenal bile of healthy volunteers after ursodeoxycholic acid intervention [29]. Enrichment of bile with ursodeoxycholic acid however has not yet been described in a group of patients after colectomy with a disturbed enterohepatic circulation.

The duodenal bile was highly cytotoxic towards the two cell lines used as test systems here, however no differences were observed in cytotoxicity of bile sampled before and after the ursodeoxycholic acid intervention. However, since trypsin present in the duodenal bile might disturb proper functioning of the cytotoxicity test and as trypsin is also used to release cells adhered to the plastic, heat inactivated bile was also tested. Heat inactivation of the trypsin in duodenal bile did indeed significantly lower the cytotoxicity and unmask the lower cytotoxicity of ursodeoxycholic acid-enriched heat inactivated bile, at least in the HT-29 cell test system. This also implies that pancreatic enzymes, and more especially trypsin, may play an important role in the cytotoxicity, either by being extremely cytotoxic to cells not protected by mucins, or by disturbing proper functioning of the cytotoxicity test by cell release from the plastic and subsequent loss of viable cells in the WST-1 assay. This has to be further investigated.

In contrast to HT-29 cells, LT97 did not reveal any difference when treated with pre- or post-treatment bile. Overall, the LT97 cells were found to be less sensitive towards the cytotoxic effects of duodenal bile, heat inactivated or not. An explanation for this is not yet available.

The lowering effect of the ursodeoxycholic acid-enriched bile on cytotoxicity might be explained by the decreased concentration of total bile acids in the post-intervention duodenal bile. It is known that ursodeoxycholic supplementation can lead to increased fasting and residual gall bladder volume [30]. Furthermore, the pancreatic secretion of amylase can be reduced by ursodeoxycholic acid when given intraduodenally [31]. In this study, there was no difference in the amylase activity before and after the intervention.

Although the duodenal bile was cytotoxic to HT-29 cells, DNA damage was not generated in these cells by heat inactivated duodenal bile. These findings seem in contrast with earlier reports where bile acids were shown to generate damage of DNA in HT-29 cells [32, 33]. However, these studies were carried out with deoxycholic acid, a secondary bile acid, which is formed in the large intestine by the bacterial 7-α dehydroxylation of cholic acid. In this study, all

patients with FAP had undergone a colectomy, and therefore had low levels of deoxycholic acid and other secondary bile acids in their enterohepatic circulation (see Figure 1).

In accordance with results of Brosens *et al.* [34], we found high expression of COX-2 in the normal duodenum of patients with FAP, sampled before the intervention with ursodeoxycholic acid. After the intervention, 4 of the 5 patients showed a less intense duodenal staining of COX-2. Probably due to the small number of patients in this pilot study, this finding is not statistically significant. Therefore, further studies on lowering the high expression of COX-2 present in the normal duodenum of these patients seems justified, especially since ursodeoxycholic acid is well tolerated and lowers cytotoxicity of bile.

It is not clear from this study whether ursodeoxycholic acid prevents development or wheter it decelerates progression of adenomas. A double-blind intervention with a larger study population should be carried out in order to elucidate this. In such at larger study, the intervention period should be lengthened and the number, size and grade of dysplasia of the duodenal adenomas should be taken as end points.

Due to germline mutations, the normal duodenal epithelial cells of patients with FAP bear one allele with loss of the *APC* gene. As a consequence, the normal duodenum of these patients has shown a higher proliferation index [35, 36], increased levels of COX-2 [34] and loss of extracellular E-cadherin [37]. In combination with the cytotoxic duodenal bile, these cellular abnormalities in the normal duodenal mucosa of patients with FAP might explain their high susceptibility to developing adenomas in the peri-ampullary region.

In conclusion, this is the first intervention study with ursodeoxycholic acid in patients with FAP. Enrichment of bile with urosdeoxychlolic acid does show a positive effect on *in vitro* cytotoxicity and seems to lower the duodenal COX-2 expression. Since pancreatic enzymes may also show considerable cytotoxicity, a double-blind controlled intervention study in patients with FAP

should be applied with a combination of ursodeoxycholic acid and inhibitors of pancreatic enzyme secretion and/or inhibitors of COX-2.

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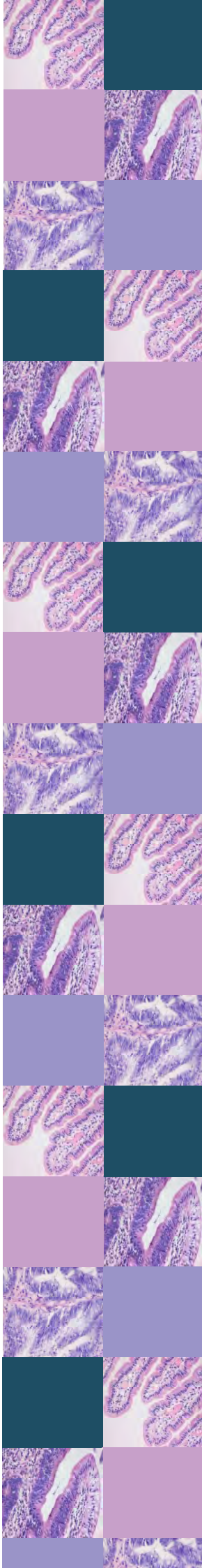
Summary



Summary

The high prevalence of duodenal adenomas in patients with FAP has been recognised for some time. The individual variety in age of onset and severity of the duodenal adenomatosis is large in patients with FAP. In contrast to the colorectal adenomatosis, the genotype-phenotype correlation as well as the exact mechanism underlying duodenal adenomatosis is much less clear for upper gastrointestinal manifestations in patients with FAP. Although duodenectomy is assumed to be an effective treatment, it is a complicated procedure with significant morbidity and even mortality. Moreover, after surgical intervention, the remaining small bowel mucosa at the choledojejunal anastomosis is at risk, since there are strong suggestions for a role of bile in FAP-associated small bowel adenomatosis. Prevention of adenoma formation by chemopreventive agents would be highly preferable in the management of the duodenal adenomatosis in patients with FAP.

In **Chapter 1**, a general introduction is given for this thesis and its objectives are summarised as follows:

1. to investigate factors that may influence the individual variety in duodenal adenomatosis in patients with FAP.
2. to further elucidate the mechanisms underlying the development of duodenal adenomas and carcinomas in patients with FAP.
3. to explore the possibility of chemoprevention of duodenal adenomas/carcinomas in patients with FAP.

In order to study interindividual variety in duodenal adenomatosis, we investigated in the first two chapters the role of the biotransformation (detoxification) enzyme families glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT) in the duodenum of FAP patients. These enzymes are important in the mucosal protection against potential mutagens and carcinogens. In **Chapter 2,** the duodenal detoxification capacity of patients with FAP was compared with that of control, matched for age and gender. GST and UGT enzyme activities and GST isoform levels were measured in both the normal duodenal mucosa of patients with FAP and that of controls. No differences were found in the detoxification enzyme levels

measured here. However, this study was performed on a limited number of patients and the assays cover only part of the GST and UGT isoenzymes present in the duodenum. We therefore performed a larger study, described in **Chapter 3**, where all GST and UGT genes of interest, covering all GST and UGT biotransformation enzymes present in the duodenum, were genotyped with respect to functional polymorphisms. A significant difference was found in the genotype distribution of *UGT1A3.* Patients with FAP more often have a genotype associated with a high UGT enzyme activity towards various flavonoids, which are constituents of fruits and vegetables. Consumption of these compounds is associated with a protection against gastrointestinal cancers. High UGT1A3 enzyme activity may result in a high rate of inactivating and excretion of flavonoids in patients with FAP compared to controls. As a consequence, the potential protective effect of flavonoids may be less pronounced in patients with FAP. Furthermore, UGT1A3 is also involved in steroid hormone metabolism. However, the polymorphisms investigated here had no predictive value for the severity of the duodenal adenomatosis in the patients with FAP.

In order to elucidate the pathways leading to the development of duodenal adenomas and carcinomas in patients with FAP, we studied the normal mucosa as well as the changes that took place after adenoma formation. The distributions of E-cadherin, SMAD4 and β-catenin in normal duodenal mucosa and adenomas in both FAP and non-FAP patients were studied as described in **Chapter 4**. Furthermore, staining of these parameters in the duodenum was compared to that in the colon. A significant loss of extracellular E-cadherin was observed in both duodenal- and colonic adenomas, as well as in normal tissue of patients with FAP. Loss of duodenal extracellular E-cadherin was associated with a younger age of the FAP patients at the moment of colectomy, suggesting a more severe course of the disease. Furthermore, nuclear localisation of β-catenin was observed more often in duodenal FAP adenomas compared with non-FAP adenomas. Loss of nuclear SMAD4 was seen in the duodenum and even more in the colon of patients with FAP, as well as non-FAP patients. Loss of duodenal SMAD4 was correlated with a high Spigelman stage in patients with FAP.

Finally, we investigated the mechanism of development of adenocarcinomas in the duodenum of FAP patients by comparing the genetic makeup of both FAP-related and sporadic adenocarcinomas of the small bowel. In **Chapter 5** the chromosomal abnormalities and alterations in methylation of several genes in sporadic and FAP-related duodenal carcinomas were studied. Chromosomal imbalances were detected in the majority of both types of tumours. A significant difference in chromosomal imbalances between the FAP-related and sporadic tumours was seen on chromosome 12. In 60% of the sporadic tumours, gains in particular regions of chromosome 12 were observed versus none in the FAP-related tumours. Identification of the genes in those regions of chromosome 12 could reveal differences in tumour characteristics and lead to a better understanding of the carcinogenesis pathways leading to sporadic and FAP-related duodenal tumours. Hypermethylation was seen in *IGSF4*, *TIMP3*, *ESR1*, *APC*, *CDH13* and *PAX6*. Hypermethylation of *PAX6* was only observed in FAP-related tumours and not in sporadic tumours.

Although duodenectomy is assumed to be an effective treatment in patients with FAP, it is a complicated procedure with significant morbidity and even mortality. Moreover, after surgical intervention, the remaining small bowel mucosa at the choledojejunal anastomosis is at risk. There are strong suggestions for a promoting role of bile in FAP-associated small bowel adenomatosis. Bile may be highly cytotoxic and may stimulate duodenal cell proliferation. Therefore, we wanted to study the effects of an intervention with ursodeoxycholic acid in patients with FAP. Ursodeoxycholic acid (UDC) is a less cytotoxic tertiary bile acid with chemopreventive potential in the treatment of several gastrointestinal manifestations. UDC (25mg/kg body weight daily) was given during three months to five patients with FAP and pre- and post-intervention bile was sampled from these patients (**Chapter 6**). Cytotoxicty of the UDC-enriched bile was approximately twice as low compared to the pre-intervention bile, whereas duodenal COX-2 expression level was not significantly reduced. No differences in genotoxicity were observed between the pre- and post-intervention bile. The results of this small pilot study warrant a more extensive study on chemoprevention with UDC in patients with FAP.

Discussion

This thesis describes investigations on several aspects of the development of adenomas and carcinomas in the duodenum of patients with FAP. Since FAP is a rare disease, most of the studies performed have included only a limited number of patients. Therefore, in future studies, a multi-centre approach should be pursued, leading to enlarged study populations and subsequently an increased power.

Several findings in this thesis underline that mechanistical differences exist between the development of adenomas and carcinomas in the duodenum and the colon. In Chapter 2, no difference was found between the detoxification capacity in the duodenum of patients with FAP versus controls. In contrast, a lower detoxification capacity was observed in the colon of patients with FAP. Also the prevalence of adenomas or carcinomas and the age of onset of the disease differ between these two locations. Figure 2 of the introduction demonstrates that *APC* and *K-ras* mutations in duodenal adenomas and carcinomas occur relatively less frequent, whereas these mutations are commonly seen in colorectal cancers of patients with FAP.

Mutations in the *APC* gene are not often found in sporadic small bowel carcinomas. However, sporadic duodenal tumours may also have a disturbed Wnt signalling pathway, possibly due to gains on chromosome 12, where the genes of the Wnt family are localised (Chapter 5).

It is likely that the *APC* mutations, as often found in patients with FAP, lead to several cellular abnormalities as described in Figure 2 of the introduction. The presence of such cellular abnormalities in “normal” mucosa, in combination with the features of the bile, may explain the high rates in the malignant transformation observed in the peri-ampullary region of patients with FAP. Interestingly, results in Chapter 6 obtained with the duodenal bile, showed a possible role for UDC in the chemoprevention of duodenal adenomatosis. The patients with FAP reported little side effects from the high dose of UDC, which makes UDC a possible candidate to be applied in such studies. However the

exact role of the pancreatic enzymes, namely their high cytotoxicity in our test system, need to be further investigated.

The non-significant reduction of cyclooxygenase-2 (COX-2) expression, as demonstrated in Chapter 6, has been shown before to be an important target for chemoprevention studies in patients with FAP. However, the use of specific and non-specific COX-2 inhibitors has been associated with upper abdominal bleedings and cardiovascular side effects. The patients with FAP reported little side effects from the high dose of UDC. Since there are many FAP patients with early duodenal adenomatosis, there is an urgent demand for chemoprevention. The lack of serious side effects makes UDC a possible candidate to be applied in such studies.

Future perspectives

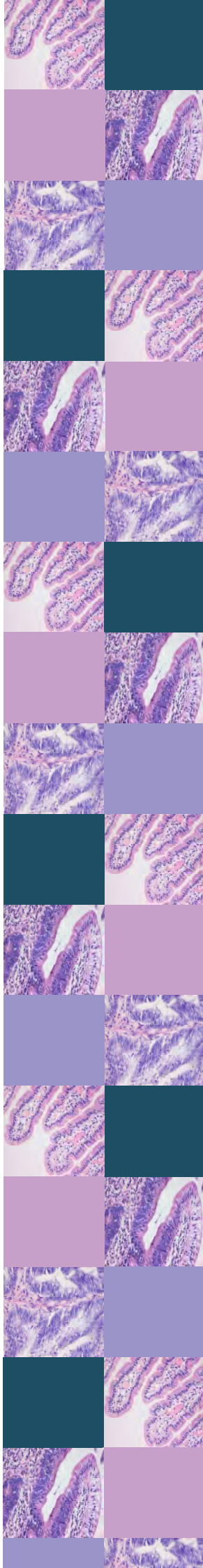
The results with ursodeoxycholic acid (UDC) in the pilot study on patients with FAP are a good basis for future research. A multi-centre randomised double-blind intervention study with UDC should be performed. Also combinations with UDC and other drugs should be explored e.g., specific COX-2 inhibitors like celecoxib, pancreas enzyme inhibitors or the flavonoids curcumin and quercetin, which were recently shown to have an inhibiting effect on ileal adenomas. In these intervention studies, regression of duodenal adenomas should be taken as primary end points.

Future research to identity genes that are located in the regions of chromosome 12, which differ between sporadic and FAP-related duodenal carcinomas, would be interesting to further elucidate mechanistical differences between both tumours. Furthermore, a comparison could be made with respect to the hypermethylation profiles of FAP-related duodenal and colorectal tumours to investigate differences between these two sites. The relatively small studies on FAP-related duodenal tumours as performed here, should be extended including carcinomas of all parts of the small intestine, as well as tumour specimens of other high-risk patients.

Conclusions

In conclusion, we believe that the chemoprevention of the duodenal adenomatosis in patients with FAP will become of great importance. Knowledge of duodenal adenomatosis is essential for the management and the treatment of these adenomas. This thesis provides new insights into the development of adenomas and carcinomas in the duodenum of patients with FAP. Furthermore, first results with an ursodeoxycholic acid intervention in patients with FAP seem promising and can hopefully provide, eventually in combination with other agents, a powerful chemopreventive treatment regimen in the future.

Samenvatting



Samenvatting

Familiaire adenomateuze polyposis (FAP) wordt veroorzaakt door mutaties in het *adenomateuze polyposis coli* (*APC*) gen. De ziekte kenmerkt zich door de ontwikkeling van honderden adenomen (poliepen) in de dikke darm. Wanneer de dikke darm niet preventief wordt verwijderd, is de kans op het ontwikkelen van dikke darmkanker bijna 100%. Ook de ontwikkeling van adenomen in de dunne darm (in het bijzonder het duodenum) van FAP patiënten is een groot probleem. Deze problematiek zal steeds meer toenemen, omdat FAP patiënten steeds ouder worden als gevolg van de preventieve verwijdering van de dikke darm. Hierdoor neemt de kans op duodenum adenomen en vervolgens het ontstaan van duodenum kanker aanzienlijk toe.

Er is echter een grote variatie tussen FAP patiënten qua mate van de adenoomvorming en de leeftijd, waarop deze duodenum adenomen ontstaan. De plaats van de mutatie binnen het *APC* gen is geassocieerd met de mate van de ontwikkeling van adenomen in de dikke darm, maar deze relatie is minder duidelijk in het duodenum. Over het mechanisme van de ontwikkeling van adenomen in de dunne darm is weinig bekend. Het verwijderen van het duodenum is een effectieve behandeling voor het voorkomen van duodenum kanker, deze zware operatie gaat echter gepaard met een hoge morbiditeit en mortaliteit. Gezien het feit dat gal een rol lijkt te spelen in de ontwikkeling van duodenum adenomen, zou het resterende dunne darm segment (jejunum) na de operatie ook weer risico kunnen lopen op maligne transformatie. Daarom zou het een groot voordeel zijn om de duodenum adenomen van FAP patiënten chemopreventief te kunnen behandelen.

In **Hoofdstuk 1** wordt een algemene inleiding gegeven en worden de doelen van dit proefschrift als volgt samengevat:

1. onderzoek naar mogelijke factoren die de variatie in de ontwikkeling van duodenum adenomen in FAP patiënten beïnvloeden.
2. onderzoek naar de mechanismen die verantwoordelijk zijn voor de ontwikkeling van duodenum adenomen in FAP patiënten.
3. onderzoek naar de mogelijkheden van chemopreventie van duodenum adenomen/ carcinomen in FAP patiënten.

Om de individuele variatie in de ontwikkeling van de duodenum adenomen te onderzoeken, worden in de eerste twee hoofdstukken de ontgiftingsenzymen glutathion S-transferasen (GST) and UDP-glucuronosyltransferasen (UGT) in het duodenum van FAP patiënten onderzocht. Deze enzymen zijn betrokken bij bescherming van de mucosa tegen potentiële mutagenen en carcinogenen. In **Hoofdstuk 2** wordt de ontgiftingscapaciteit van het duodenum van FAP patiënten vergeleken met die van proefpersonen zonder FAP. Naast de GST en UGT enzymactiviteit zijn verder zijn ook vier GST isovormen gekwantificeerd in beide groepen. Er wordt geen verschil gevonden tussen FAP duodenum en controle duodenum, wat betreft de gemeten ontgifitingsenzymen. Het aantal FAP patiënten was echter relatief laag, terwijl ook maar een deel van de GSTs en UGTs is bepaald door de hier gebruikte methoden. We hebben daarom in **Hoofdstuk 3** van een grotere groep FAP patiënten het genotype bestudeerd van alle belangrijke isovormen van de GST en UGT ontgiftingsenzymen, die aanwezig zijn in het duodenum. Er werd een significant verschil gevonden in de verdeling van het *UGT1A3* genotype. De FAP patiënten hebben vaker het *UGT1A3* genotype, dat geassocieerd is met een verhoogde enzymactivieit ten opzichte van flavonoïden. Flavonoïden komen voor in groente en fruit en ze lijken een beschermende werking te hebben tegen de ontwikkeling van verschillende kankers van het maagdarmkanaal. Als gevolg van het snelle genotype worden de flavonoïden sneller geïnactiveerd in FAP patiënten. Dit zou er toe kunnen bijdragen dat deze patiënten minder baat hebben bij de beschermende werking van flavonoïden. Verder zijn de UGT1A3 enzymen ook betrokken bij de steroïd hormoon huishouding. Deze onderzochte polymorfismen blijken echter geen voorspellende waarde voor de ontwikkeling van duodenum adenomen te hebben.

Om het mechanisme van de ontwikkeling van adenomen en carcinomen in het duodenum van FAP patiënten te bestuderen, hebben we bepaalde eigenschappen van de normale mucosa vergeleken met die van adenomen. In **Hoofdstuk 4** is er gekeken naar de aankleuring van E-cadherine, β-catenine en SMAD4 in normaal en adenomateus duodenum weefsel van FAP en niet-FAP patiënten. Ook zijn de aankleuringspatronen in het duodenum vergeleken

met de dikke darm. Er is een significant verlies gevonden van extracellulair E-cadherine in adenomen en normaal weefsel van zowel het duodenum als de dikke darm van patiënten met FAP. Dit verlies is geassocieerd met een (jongere) leeftijd, waarop de colectomie is uitgevoerd, wat op een ernstiger fenotype zou kunnen duiden. β-catenine bevindt zich vaker in de celkernen in FAP adenomen vergeleken met niet-FAP adenomen. In zowel FAP als niet-FAP patiënten werd een verlies van nucleair SMAD4 gevonden in het duodenum en zelfs nog meer in de dikke darm. In het duodenum was dit verlies van SMAD4 geassocieerd met een hoger Spigelman stadium, m.a.w. een meer ernstigere vorm van duodenum adenomen.

Als laatste hebben we gekeken naar de ontwikkeling van carcinomen, door de genetische opmaak van sporadische duodenumcarcinomen te vergelijken met die van FAP-gerelateerde carcinomen. Daarom zijn in **Hoofdstuk 5** chromosomale afwijking en veranderingen in de methylering van verschillende genen bestudeerd, in zowel sporadische als FAP-gerelateerde duodenumcarcinomen. In de meeste tumoren werden chromosomale afwijkingen gevonden. Vooral interessant is het significante verschil tussen sporadische en FAP-gerelateerde carcinomen met betrekking tot chromosoom 12. In 60% van de sporadische carcinomen werd een amplificatie gevonden in bepaalde gebieden op chromosoom 12, terwijl in de FAP-gerelateerde carcinomen deze afwijkingen nooit werden gevonden. Het identificeren van de genen, die zich in deze gebieden bevinden, zou daarom tot meer kennis kunnen leiden over de ontwikkeling van zowel sporadische als FAP-gerelateerde carcinomen. Ook werd hypermethylatie van de genen *IGSF4*, *TIMP3*, *ESR1*, *APC*, *CDH13* en *PAX6* in de duodenumcarcinomen gevonden. Hypermethylatie van het gen *PAX6* werd alleen gezien in de FAP-gerelateerde carcinomen.

Gal lijkt een rol te spelen bij de formatie van adenomen in het duodenum van FAP patiënten, onder meer omdat deze adenomen vooral rondom de papil van Vater (galafvloed) geconcentreerd zijn. Het veranderen van de galsamenstelling (minder cytotoxisch maken) is een mogelijke vorm van chemopreventie. Ursodeoxycholzuur (UDC) is een weinig cytotoxisch tertiair

galzuur met chemopreventieve eigenschappen. Daarom hebben we in **Hoofdstuk 6** onderzocht of een hoge dosis van UDC wordt verdragen door FAP patiënten, die geen dikke darm meer hebben. Gedurende 3 maanden zijn vijf patiënten behandeld met UDC (25 mg/kg eenmaal daags). De cytotoxiciteit en genotoxiciteit zijn gemeten van de gal, die voor en na de interventieperiode verzameld is. Uit deze studie blijkt dat UDC goed wordt verdragen door FAP patiënten en dat hun gal aanzienlijk is verrijkt met dit tertiaire galzuur. Na hitte inactivatie van de pancreasenzymen, die ook aanwezig zijn in duodenum gal, bleek de cytotoxiciteit van de gal na de interventie significant lager dan voor de interventie. Ook bleek de cyclooxygenase-2 (COX-2) expressie, een sleutelenzym voor de regulering van de celdeling, verminderd na de UDC interventie (niet-significant). De genotoxiciteit bleek niet verschillend tussen de gal van voor en na de UDC interventie. De resultaten van deze kleine pilot studie zijn interessant genoeg om UDC te testen als een chemopreventief middel in een grotere groep FAP patiënten.

Discussie

In dit proefschrift worden verschillende studies beschreven met betrekking tot de ontwikkeling van adenomen en carcinomen in het duodenum van FAP patiënten. FAP is een zeldzame aandoening, in Nederland zijn ongeveer 600 patiënten bekend. Dit is meteen de reden dat de studies in dit proefschrift vaak op een klein aantal patiënten zijn gebaseerd. Toekomstig onderzoek m.b.t. FAP patiënten zou daarom ook gebaat zijn bij een multi-center aanpak, hierdoor zouden grotere patiënten populaties bestudeerd kunnen worden, waardoor de statistische power vergroot zou worden.

Meerdere bevindingen uit dit proefschrift wijzen op een verschillend mechanisme van adenoomvorming in het duodenum ten opzichte van dat in de dikke darm. In Hoofdstuk 2 werd geen verschil gevonden in de ontgiftingscapaciteit van het duodenum van FAP patiënten ten opzichte van controles, terwijl dat eerder wel in de dikke darm werd gevonden. Verder is er ook een verschil tussen deze twee locaties in de prevalentie van adenomen en carcinomen en de leeftijd waarop de adenoomvorming begint. Figuur 2 uit

de Introductie laat zien dat *APC* en *K-ras* mutaties minder vaak voorkomen in het duodenum van FAP patiënten in vergelijking met de dikke darm.

APC mutaties worden minder vaak gevonden in sporadische dunne darm carcinomen. Mogelijk hebben sporadische duodenum carcinomen echter toch een verstoring in de Wnt signaalroute door de gevonden amplificaties in de regio's op chromosoom 12, waar de genen van het WNT signaalroute zich bevinden (Hoofdstuk 5).

Het is aannemelijk dat de *APC* mutaties, zoals die vaak in FAP patiënten gevonden worden, leiden tot de cellulaire afwijkingen, zoals beschreven in Figuur 2 van de introductie. Vooral de afwijkingen die zich al in "normale" mucosa bevinden, zouden in combinatie met meer cytotoxische gal, een verklaring kunnen zijn voor het hoge risico van FAP patiënten op een maligne transformatie in het gebied rond de papil van Vater. Uit Hoofdstuk 6 blijkt echter ook dat de pancreasenzymen uit duodenumgal een hoge cytotoxiciteit vertonen met onze meetmethode. Onduidelijk is daarom nog in hoeverre deze pancreasenzymen cytotoxisch zijn *in vivo* en daarom lijkt het zinvol om de rol van pancreasenzymen in de ontwikkeling van adenomen verder uit te zoeken.

De niet-significante vermindering van COX-2 expressie in het duodenum, zoals te zien in Hoofdstuk 6, kan ook een belangrijk aangrijpingspunt zijn voor chemopreventie studies bij FAP patiënten. Het gebruik van specifieke en aspecifieke COX-2 remmers is echter geassocieerd met een hoger risico op bloedingen in het maagdarmkanaal en ook op cardiovasculaire bijwerkingen. Het gebruik van een hoge dosis van UDC leidde echter tot weinig bijwerkingen bij de FAP patiënten. Er zijn veel FAP patiënten met een beginnende vorm van adenoomvorming, waardoor een chemopreventief middel erg gewenst is. UDC zou, eventueel in combinatie met andere geneesmiddelen, een goede kandidaat kunnen zijn voor chemopreventie studies in FAP patiënten.

Toekomstig onderzoek

De resultaten van de pilot studie met UDC bij FAP patiënten zijn een goede basis voor toekomstig onderzoek. Daarom zou een multi-center,

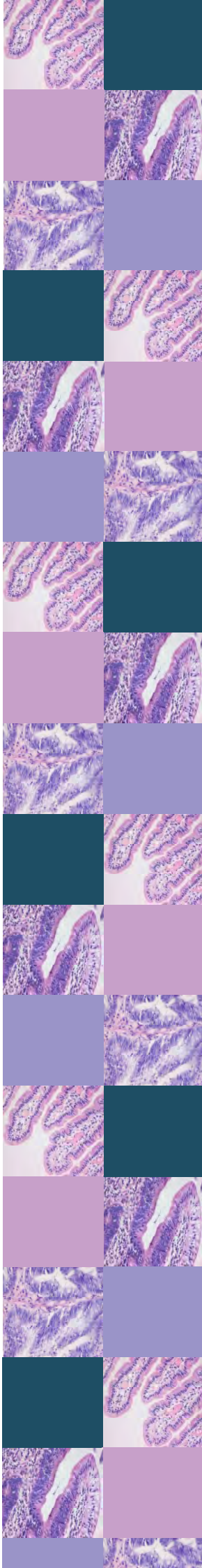
gerandomiseerde dubbel-geblindeerde interventie studie moeten worden uitgevoerd, waarin verschillende combinaties geneesmiddelen samen met UDC zouden moeten worden getest: bijv. de specifieke COX-2 remmer celecoxib, remmers van pancreasenzymen of de flavonoïden curcumine en/of quercitine. Deze flavonoïden hebben recent een remming van de adenoomgroei in het ileum laten zien. In deze interventie studies zou de regressie van duodenum adenomen als primair eindpunt moeten worden genomen.

Verder onderzoek naar de verschillen in genen op chromosoom 12, zoals te vinden in sporadische en FAP-gerelateerde duodenumcarcinomen, zou het mechanisme achter de carcinoomvorming meer kunnen verhelderen. Omdat hypermethylering van genen een belangrijke rol kan spelen in het onstaan van carcinomen, zou een meer uitgebreide vergelijking moeten worden gemaakt tussen de hypermethyleringsprofielen van dunne darm en dikke darm carcinomen. De relatief kleine studies van FAP-gerelateerde duodenumcarcinomen zoals beschreven in dit proefschrift, zouden uitgebreid moeten worden met carcinomen van alle delen van de dunne darm en ook met tumoren van andere patiënten groepen, die een verhoogd risico hebben op het ontstaan van dunne darm kanker.

Conclusies

Adenoom- en carcinoomvorming in het duodenum van FAP patiënten zal met het toenemen van de leeftijd van deze patiënten een steeds groter probleem worden. Kennis over het mechanisme van deze adenoom- en carcinoomvorming is essentieel voor de behandeling van deze tumoren. Dit proefschrift geeft nieuwe inzichten in de ontwikkeling van adenomen en carcinomen in het duodenum van FAP patiënten. Chemopreventie zou de behandeling van de toekomst kunnen worden en de eerste resultaten van de ursodeoxycholzuur interventie in FAP patiënten lijken veelbelovend.

Dankwoord



“Rondje Radboud”

Wekker. Altijd weer te vroeg! Naar beneden met de katten achter me aan voor ons ochtendritueel. Over de Rijn, over de Waal en ik ben weer in het Radboud. Daar kom ik meestal als eerste bij mijn kamer Prof. Jansen tegen.

Beste Jan, bedankt dat ik op jouw afdeling mocht werken en voor altijd een vriendelijk woordje. Na even op mijn kamer geweest te zijn, daal ik tot de onderverdieping naar ons lab, waar ik Hennie tref. Hennie, zonder jouw kennis en hulp had ik nooit al de experimenten kunnen afronden. Ook de andere (ex)bewoners van het lab, René, Annie, Wim, Saskia, Loes en Albert heel erg bedankt voor alle hulp, gezelligheid en voor het altijd beantwoorden van mijn vragen! Ook onze buren: lab Interne, wil ik graag bedanken.

Ik loop weer naar boven, mijn kamer, daar vind ik Esmé en Michelle. Ook jullie wil ik graag bedanken voor jullie vrolijkheid, morele ondersteuning, wetenschappelijke discussies en het altijd in zijn voor “capusjieno” drinken. Ook mijn kamergenoten uit eerdere tijden: Elise en Carolien, bedankt voor jullie gezelligheid en het delen van (statistische) kennis! Ook Mariette, wil ik graag bedanken voor alle gezelligheid van de afgelopen jaren en voor de morele steun als paranimf! De longziektenburen van onze kamer, Hanneke en Alma wil ik graag bedanken voor het delen van onderzoeksvreugde, maar ook leed!

Ik loop door over de gang en ik kom langs het secretariaat: ook hier bedankt! Verder over de MDL-gang: de laatste kamer links van Dr. Nagengast.

Beste Fokko, ik heb veel geleerd van je enthousiaste, maar altijd kritische houding t.o.v. mijn onderzoek. Dank daarvoor! Ook je kamergenoot, Maria, wil ik graag bedanken voor de altijd snelle aanlevering van gegevens, die ik weer eens nodig had. Op naar Villa Interne, de plek die ook al in 2001 bezocht als voedingsstudente en waar Dr. Peters “woont”.

Beste Wilbert, ik heb altijd heel erg fijn met je samengewerkt en ik heb veel geleerd van jouw onderzoekservaring. Je wist (meestal) mijn negatieve instelling over resultaten om te buigen naar iets positiefs. Ik verlaat de Villa Interne en loop langs de endoscopie afdeling. Daar heb ik ook veel tijd doorgebracht en ik wil dan ook alle verpleegkundigen en de artsen, die me geholpen hebben erg bedanken.

Ik verlaat het MDL-deel van het Radboud en loop over een lange gang richting de Pathologie, mijn tweede “thuis”. Prof. van Krieken, beste Han, je hebt er altijd voor gezorgd dat ik me ook echt thuis voelde op je afdeling. Onze wekelijkse werkbesprekingen zijn erg waardevol voor me geweest. Hierbij wil ik dan ook de andere leden van die werkbespreking bedanken voor hun vaak frisse inbreng. Een andere wetenschappelijke inspiratiebron is Dr. Nagtegaal voor me geweest. Beste Iris, dank je wel voor al het vertrouwen dat je altijd in me hebt gehad en voor het, waar ter wereld ook, razendsnel bekijken van mijn stukken (was ik net blij dat ik het even niet meer hoefde te zien…).
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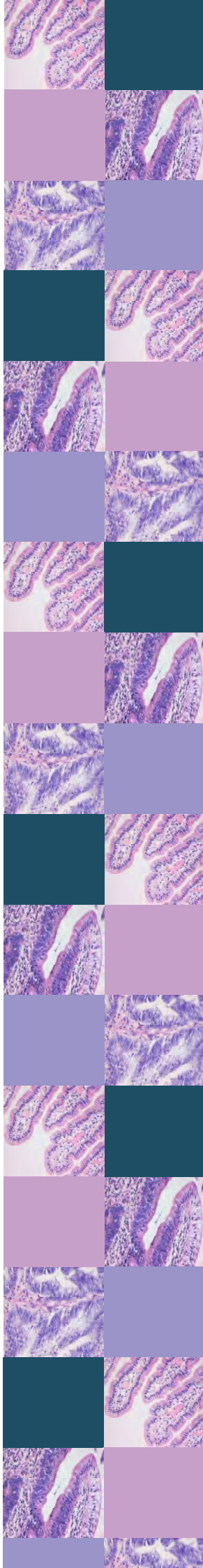
Mensen, die ik nu niet op mijn rondje ben tegengekomen, maar die ik wel graag wil bedanken voor hun enthousiasme en inzet zijn de studenten, die ik heb begeleid: Marloes, Dorien, Marleen, Katrien en Monica. Verder wil ik ook nog de mensen van “Medical Science” bedanken.

Buiten het Radboud zijn er ook een paar mensen, die ik graag wil bedanken voor hun fijne samenwerking: Fred van de Molengraft, Evelien Dekker en Guido Hooiveld. En ook vanuit Wageningen: Mariken en Gerda! De familie Scholes wil ik graag bedanken voor hun interesse en het checken van mijn manuscript.

Buiten mijn rondje zijn er nog een heel aantal mensen, die ik niet genoemd heb, maar die het wel mogelijk hebben gemaakt om mijn proefschrift af te ronden: iedereen heel erg bedankt daarvoor!

Ik vertrek weer vanuit het Radboud richting Rhenen: mijn echte thuis! Daar wil ik graag mijn vrienden (uit Den Haag & Wageningen) bedanken voor hun vriendschap en gezelligheid. Ook mijn familie en schoonfamilie wil ik graag bedanken voor hun belangstelling en steun. En Albert & Miranda, jullie ook bedankt voor alle ontspannende fietstochtjes en andere uitjes. Ik vind het ontzettend leuk om tante te worden in november. Lieve Mam en Jan, heel erg bedankt voor jullie onvoorwaardelijke steun en liefde, het lijkt misschien allemaal maar vanzelfsprekend, maar dat is het natuurlijk niet, dank jullie wel daarvoor. En tenslotte, de reden, waarom ik het echt fijn vind om thuis te komen. Dat ben jij, Rinke. Je bent de liefste en meest verrassende persoon die ik ken en ik kan me een leven zonder jou niet voorstellen.

List of publications



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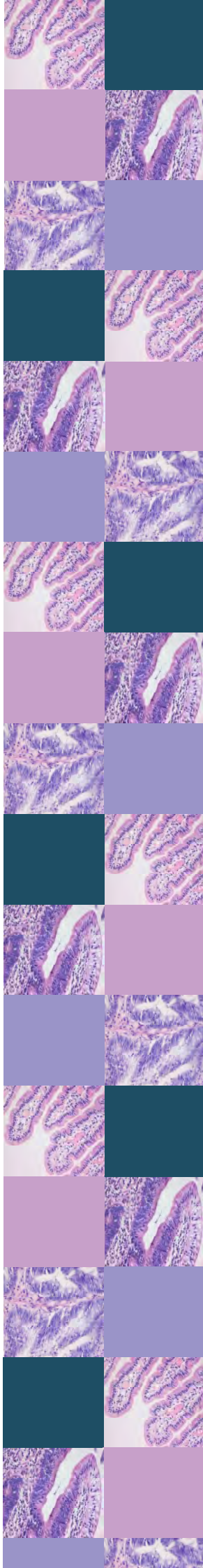
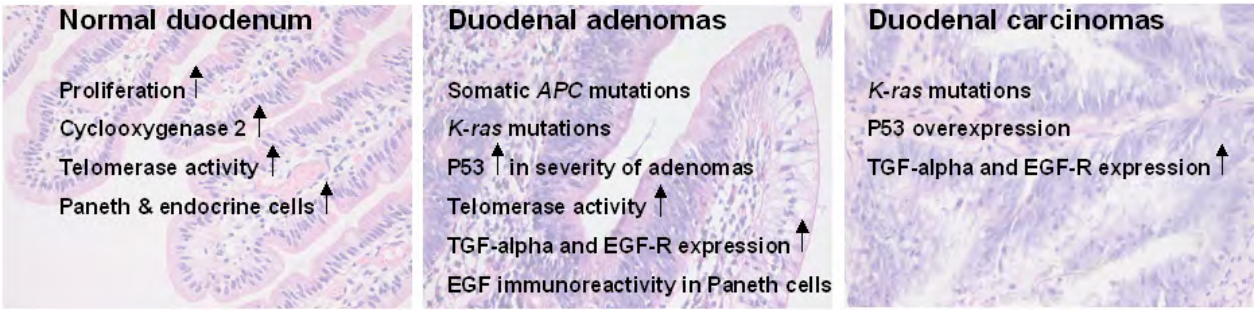
Colour figures



Chapter 1

Figure 2



Chapter 4

Figure 1

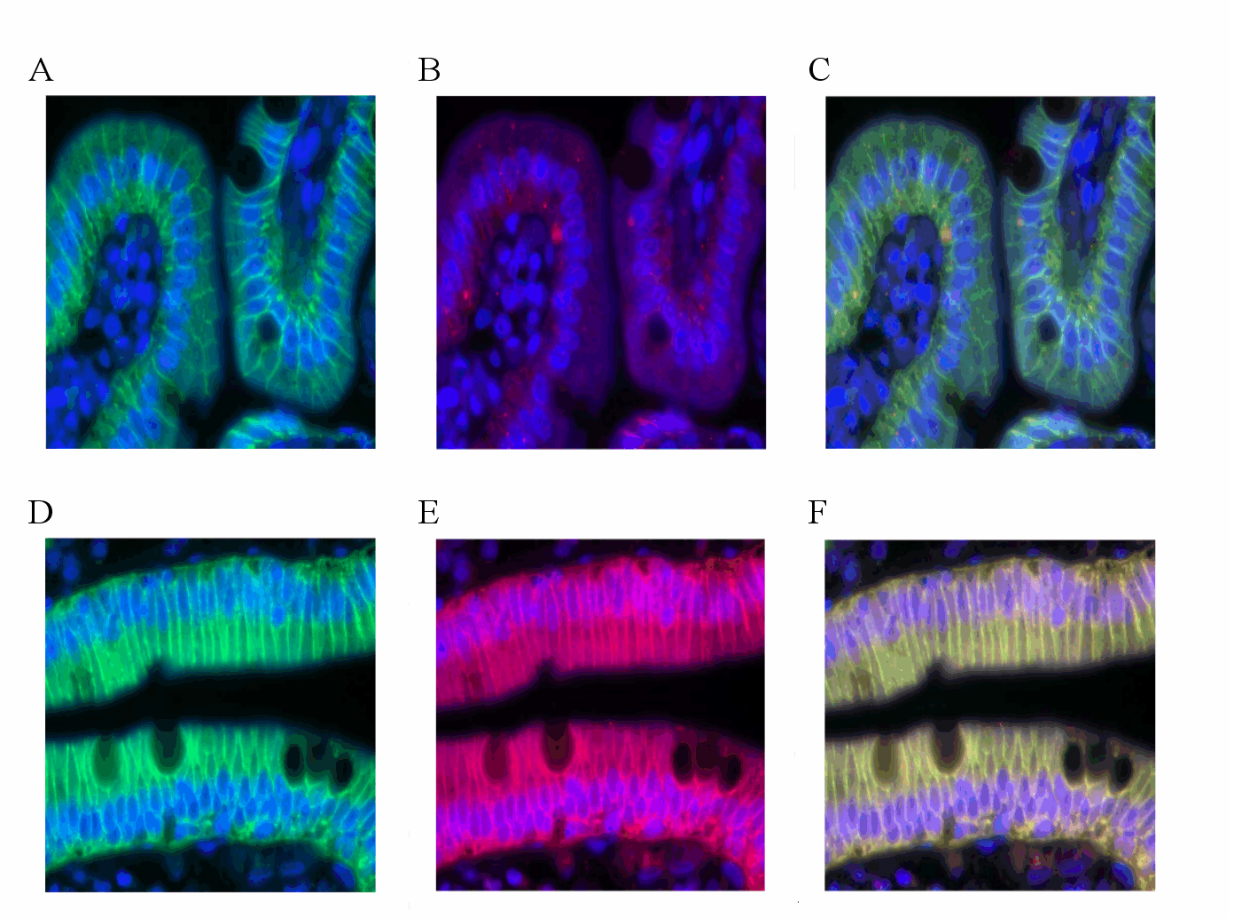


Figure 2

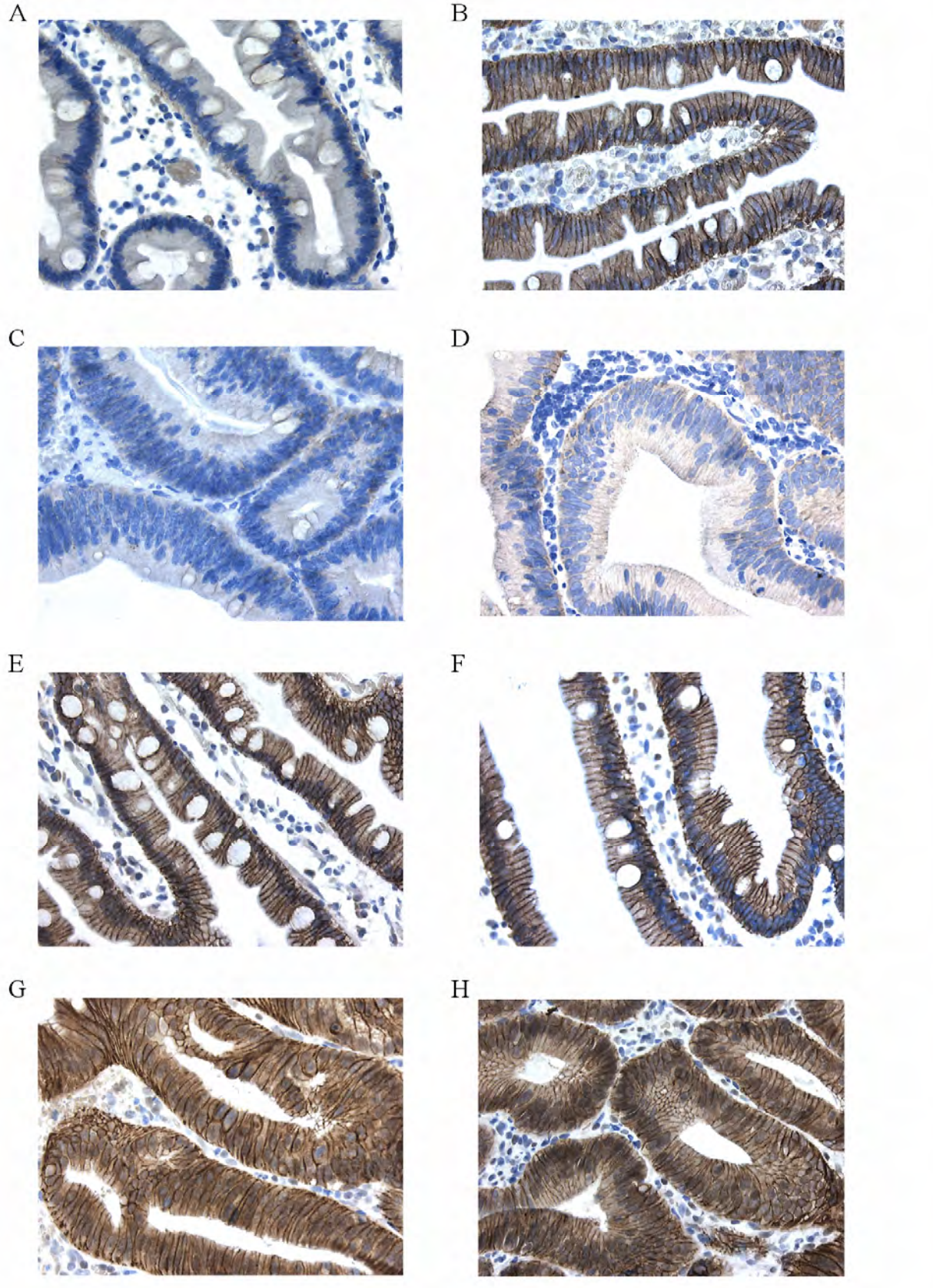
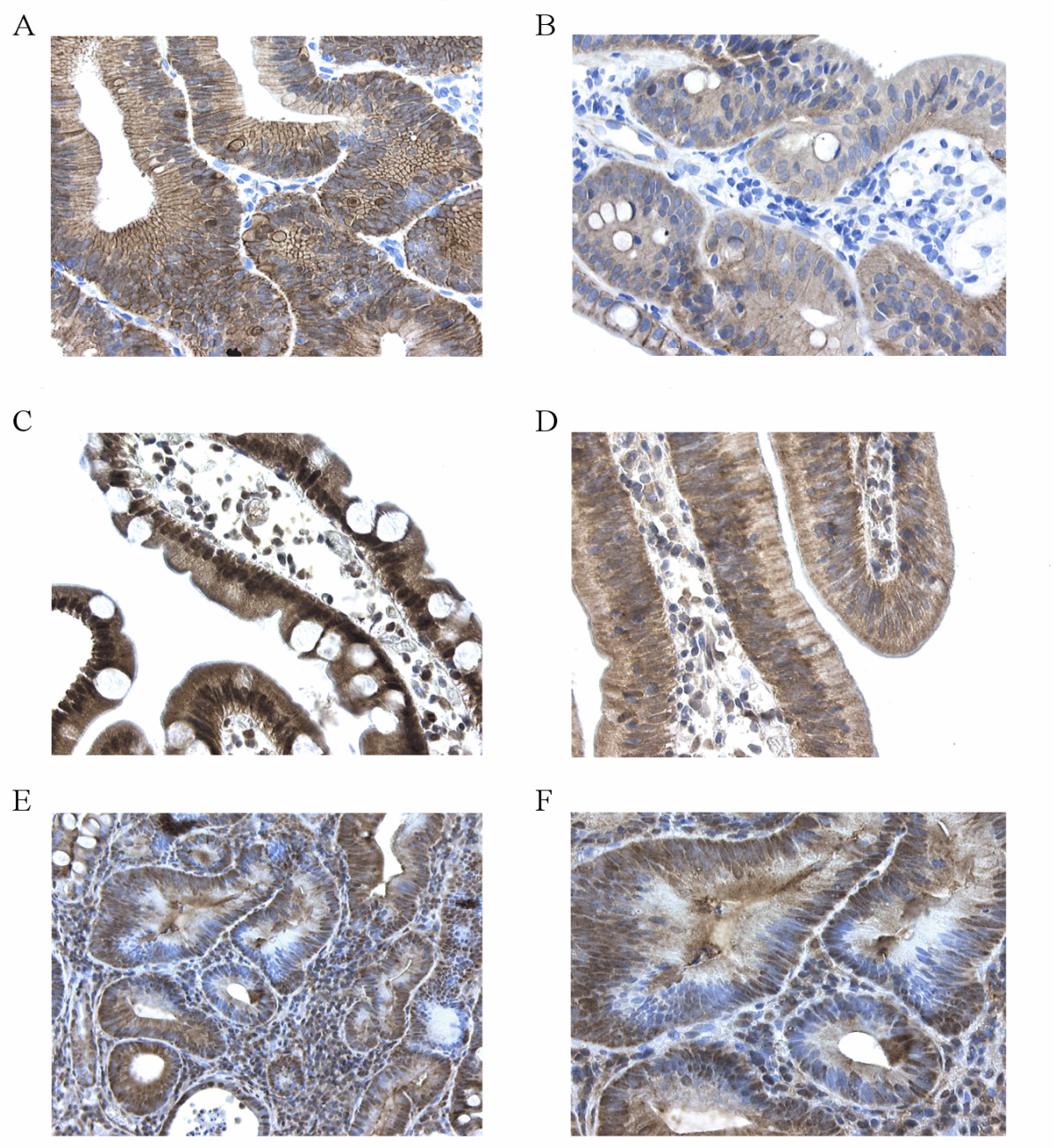
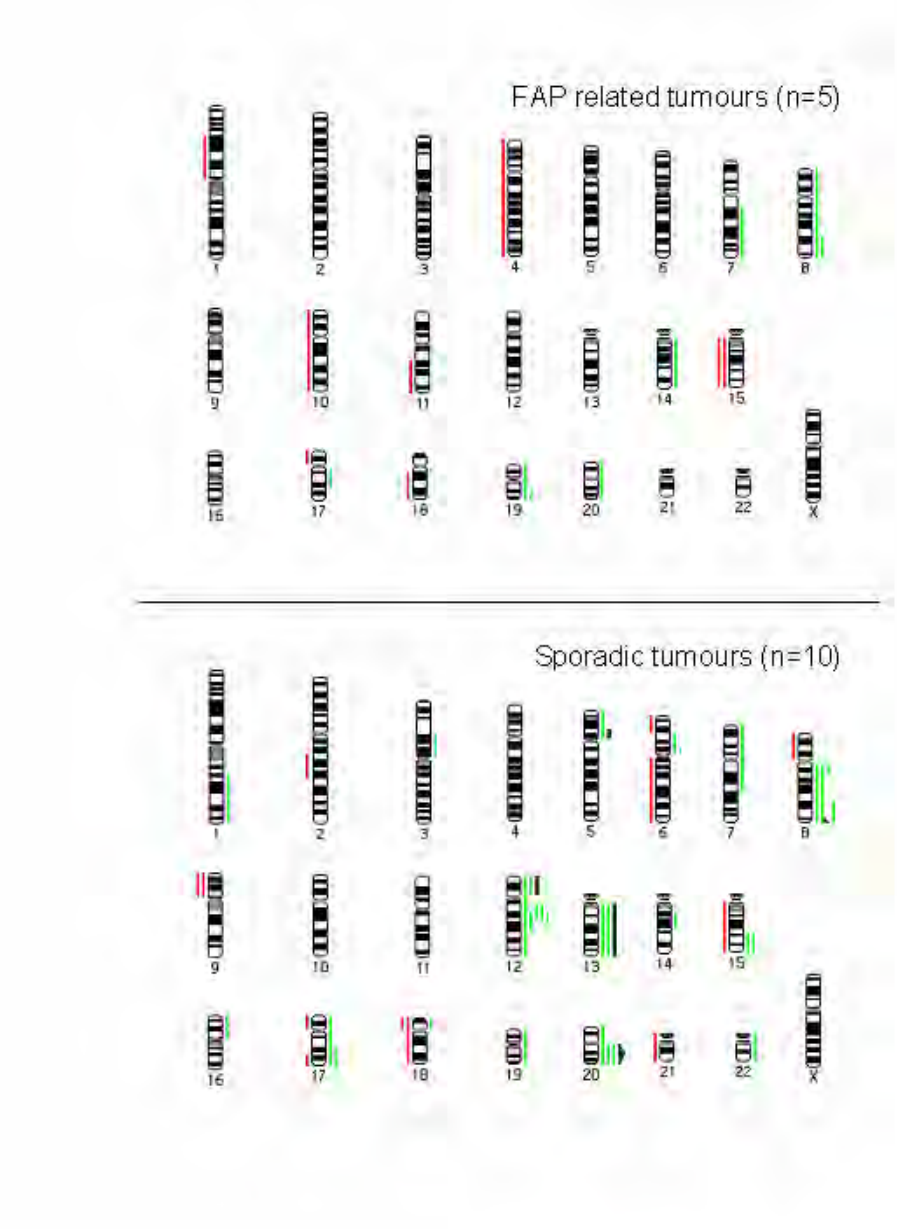


Figure 3



Chapter 5

Figure 1



Chapter 6

Figure 5